Characterisation of mammalian central nicotinic acetylcholine receptors

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CHARACTERISATION OF MAMMALIAN CENTRAL NICOTINIC ACETYLCHOLINE RECEPTORS

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for the degree of Ph.D.

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ABBREVIATIONS

ACh	Acetylcholine
AChR	Acetylcholine receptor
α-BGT	α-Bungarotoxin
Br-ACh	Bromoacetylcholine
CNS	Central nervous system
DA	Dopamine
DHBA	3,4-Dihydroxybenzylamine
DHβE	Dihydro-β-erythroidine
DMPP	1,1-Dimethyl-4-phenylpiperazinium
DMSO	Dimethylsulphoxide
DOPAC	3,4-Dihydroxyphenyl acetic acid
DPBEA	Diphenylborate-ethanolamine
EDTA	Ethylenediaminetetra-acetic acid, disodium salt
HEPES	N-2-Hydroxyethylpiperazine N'-2-ethane sulphonic acid
HPLC with ECD	High performance liquid chromatography with electrochemical detection
HTX	Histrionicotoxin (H_{12}HTX, Perhydrohistrionicotoxin)
HVA	Homovanillic acid
LDH	Lactate dehydrogenase (L-lactate: NAD^+ oxidoreductase E.C. 1.1.1.27)
mAChR	Muscarinic acetylcholine receptor(s)
MAO	Monoamine oxidase
MBTA	4-(N-maleimido)-benzyl trimethylammonium iodide
nAChR	Nicotinic acetylcholine receptor(s)
NSTX	Neosurugatoxin
PBS	Phosphate-buffered saline
PNS	Peripheral nervous system
PPO	2,5-Diphenyloxazole
PMSF	Phenylmethylsulphonylfluoride
REC	Receptor-effector complex
ToABr	Tetraoctylammonium bromide
TTX	Tetrodotoxin

SUMMARY

A synaptosomal perfusion system was developed to study a pre-synaptic nicotinic acetylcholine receptor (nAChR) which facilitates dopamine (DA) release in the striatum.

Rat striatal synaptosomes, preloaded with [^{3}H]DA, were layered onto glassfibre filters in perfusion chambers, washed with Krebs-bicarbonate medium and repeatedly stimulated. The identity of the released [^{3}H]DA was confirmed by HPLC, furthermore both basal and stimulated release were Ca^{2+}-dependent.

Collection of small (340 µl) fractions, and the use of [^{3}H] DA of high specific activity conferred high sensitivity on the system. K^+ (16 - 60 mM), veratridine (10 - 100 µM) and (-)-nicotine (0.01 µM - 1 mM) evoked [^{3}H]DA release in a dose-dependent manner. The EC_{50} value for (-)-nicotine was 3.8 µM and a 100 fold difference in the relative potency of (-)-nicotine to (+)-nicotine was demonstrated. Acetylcholine and the ganglionic agonists 1,1-dimethyl-4-phenylpiperazinium and cytisine were equipotent to (-)-nicotine. The pharmacological specificity of the nicotinic response was investigated using a range of antagonists. Neosurugatoxin, mecamylamine, pempidine and dihydro-β-erythroidine blocked the nicotinic facilitation, although α-BGT and decamethonium were less effective, suggesting that the nicotinic heteroreceptor shows characteristics of both ganglionic and neuromuscular nAChR. The nicotinic ion channel blockers perhydrohistrionicotoxin and ketamine also blocked the action of nicotine whereas tetrodotoxin was ineffective.

In parallel studies, synaptosomal membranes from striata were shown to have nicotinic binding sites measured by the specific binding of [^{3}H]nicotine and [^{125}I]α-BGT.

Preliminary experiments measuring the release of [^{3}H]ACh from hippocampal synaptosomes confirmed the presence of a nicotinic autoreceptor with similar pharmacological characteristics to the striatal heteroreceptor.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 BACKGROUND: NICOTINE

> "What is clear is that there is a mixture of stimulant and depressant properties at a variety of levels. These involve the central and peripheral nervous systems, the cardiovascular and endocrine systems. This complex of effects is further intermingled with personality and other variables that result in the complicated behaviour ... that is the phenomenon of tobacco smoking."
>
> Domino, 1979.

Nicotine is a pharmacologically active alkaloid of the tobacco plant *Nicotiana tabacum* and has been used as a stimulating agent by man for centuries(Ashton and Stepney, 1983). The use of tobacco and its associated medical problems can be attributed to the addictive properties of nicotine(Ashton and Stepney, 1983).

Nicotine is readily absorbed from the stomach after oral administration and from the lungs after inhalation. Nicotine is soluble in both water and lipid and therefore rapidly penetrates the brain and all body organs. It is metabolised (with a half-life of 2 h) mainly in the liver and kidney and also the lung, to form cotinine and nicotine-1'-N-oxide. Nicotine and its metabolites are rapidly removed from the body by the kidney (Taylor, 1985a). However, when nicotine is present in the body many behavioural and pharmacological actions have been recorded such as increased motor activity, raised blood pressure and heart rate, the release of adrenaline from the adrenal glands, and increased tone and activity of the gastrointestinal tract (Taylor, 1985b). Nicotine therefore acts at many central and peripheral sites. Although the sites at which nicotine acts in the periphery have been characterised, relatively little is known about its sites of action in the brain.

This introduction describes how nicotine was first used in the classification of acetylcholine receptors and how within the peripheral nervous system there are two pharmacologically distinct types of nicotinic receptor. This is followed by a review of the present knowledge of the biochemistry of the nicotinic acetylcholine receptor purified from peripheral sources. Finally, an account is given of the evidence for nicotinic receptors in the central nervous system.

1.1.1 The classification of cholinergic receptors

In 1905, Langley reported that low doses of nicotine stimulated and high doses paralysed autonomic ganglia (Langley, 1905). At about the same time as these observations the theory of chemical transmission was being developed. Ramon y Cajal (1888) proposed that nerve cells were discrete units, each enclosing its own cytoplasmic content and making close contact with each other. The theory of chemical transmission suggested that transfer of information from the neurone to a neuro-effector tissue (or between neurones) was achieved by the release of a chemical from the nerve terminal. This chemical then diffused across the gap between the nerve terminal and the effector tissue and combined with a recognition site. The gap separating the neurone and effector tissue was termed the synapse (Sherrington, 1925) and the recognition site, after Langley (1907) 'a receptive substance', known later as 'a receptor'.

Otto Loewi (1921) in his classical experiments on the vagal nerve stimulation of perfused frog hearts, provided the first evidence for the actual release of a chemical in response to

activation of a nerve. The chemical (or neurotransmitter) released upon vagal stimulation was later identified as acetylcholine (ACh).

There are two distinct responses to ACh; those which are mimicked by nicotine, and those which are mimicked by muscarine (from the mushroom *Amanita muscaria;* Dale, 1914). The response to nicotine is excitatory and although it was originally shown by Dale (1914) that muscarine responses were inhibitory, excitatory muscarinic responses have also been shown (see Krnjević, 1975).

Under this original classification two types of cholinergic receptors were defined, nicotinic acetylcholine receptors (nAChR) and muscarinic acetylcholine receptors (mAChR). The action of nicotine is characterised by a fast response (1 - 2 ms) and is blocked by excess nicotine or by d-tubocurarine. In contrast, the muscarinic response tends to be slow in onset and prolonged (~ 100 ms) and is blocked by atropine.

1.1.2 Neuromuscular transmission

An early account of the molecular events of cholinergic transmission at the neuromuscular junction was provided by Katz (1966). Described below is a more recent account based on this work.

An action potential initiated in the cell body of a motor neurone passes along the axon and invades the nerve terminal. This temporary depolarisation of the nerve ending opens voltage-sensitive Ca^{2+} channels and leads to an influx of Ca^{2+}. The release of ACh is triggered either directly by the sudden increase in intracellular Ca^{2+} (Blaustein, 1979; Silinsky, 1985) or by a

calcium/calmodulin-dependent protein kinase (DeLorenzo, 1982). The excess Ca^{2+} is taken up by intracellular Ca^{2+} pools such as mitochondria or the endoplasmic reticulum and the final steady Ca^{2+} balance is re-established by means of a transmembrane Na^{+}/Ca^{2+} exchange (Blaustein, 1979). The ACh released into the synaptic cleft diffuses across to the post-synaptic membrane and binds to the nAChR. This results in a conformational change in the receptor and the opening of an associated ion-channel. Na^{+} and K^{+} ions flow through the channel down their respective electrochemical gradients, with more Na^{+} ions passing in that K^{+} moving out, resulting in a net influx of positive charge. This local depolarisation produces an 'end-plate potential' (E.P.P.). If the summation of E.P.P.s causes depolarisation to the threshold value then an action potential is generated which is propagated along the muscle fibre and causes contraction. The 'quantal' theory of chemical transmission was formulated after the discovery of spontaneous miniature end-plate potentials (M.E.P.P.s; Katz, 1969). M.E.P.P.s are the result of the random release of 'packets' of ACh from the motor nerve terminal which interact with the receptors at the end-plate. Such packets, termed quanta, were considered to represent a discrete, minimum amount of ACh that could be released from the presynaptic terminal. The isolation of synaptic vesicles from nerve terminals (De Robertis *et al.*, 1962; Whittaker *et al.*, 1964) was taken in support of the quantal theory in that individual vesicles were considered to represent quanta but such speculations have never been substantiated experimentally. However, the exact mechanism of ACh release is still controversial and release is

considered by some groups to be from cytoplasmic rather than vesicular pools (Dunant and Israel, 1985).

Studies have also shown that there is another type of ACh release at the neuromuscular junction. This release is Ca^{2+}-independent, non-quantal in nature and consists of molecular leakage of ACh from the nerve terminal (Katz and Miledi, 1977). This efflux is not part of normal synaptic transmission and although it is quantitatively large its physiological role is unknown.

1.1.3 The vertebrate nervous system and the distribution of cholinergic receptors

In the vertebrate, the nervous system is divided into two main parts, the central nervous system (CNS) comprising the brain and spinal cord, and the peripheral nervous system (PNS) which is made up of all the nervous system that is outside the CNS. The PNS is further subdivided into the somatic nervous system (which is made up of the voluntary motor nerves to skeletal muscle, and sensory nerves) and the autonomic nervous system which innervates the muscle, glands and blood vessels of the internal body organs. A further functional and anatomical subdivision of the autonomic nervous system can be made to define the sympathetic and the parasympathetic nervous systems. The main neurotransmitter in the sympathetic nervous system is noradrenaline whereas in the parasympathetic nervous system ACh is the major transmitter at the end organ.

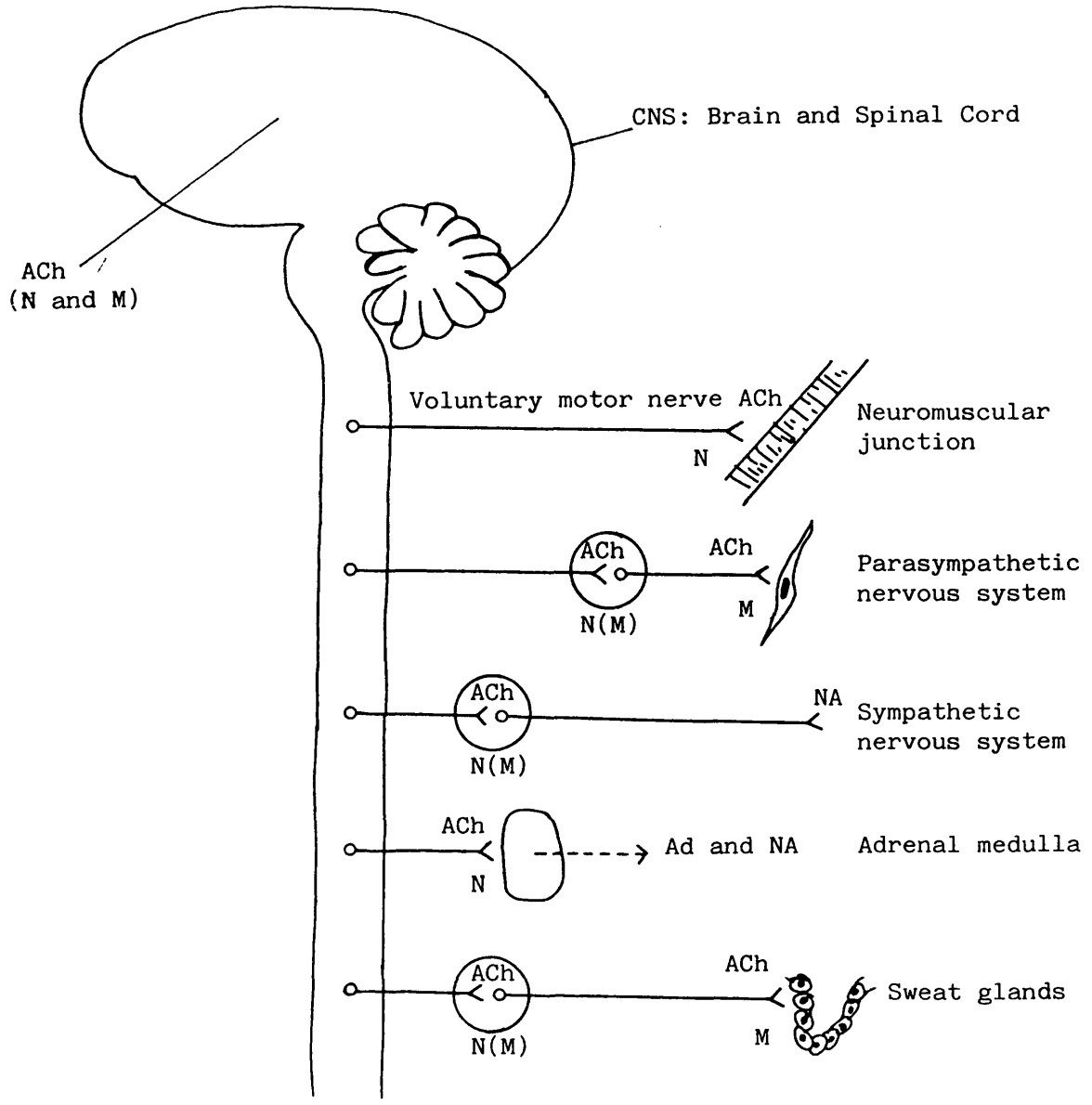


Fig. 1.1. Location of cholinergic synapses in the nervous system.

N : Postsynaptic nicotinic receptors

M : Postsynaptic muscarinic receptors

Ad: Adrenaline release from adrenal medulla

NA: Noradrenaline release from sympathetic nerve ending.

Fig. 1.1 shows the main sites at which ACh acts in the vertebrate nervous system. These can be summarised as follows:-

1. Autonomic effector sites, innervated by postganglionic parasympathetic fibres.
2. Sympathetic and parasympathetic ganglion cells and the adrenal medulla, innervated by preganglionic autonomic fibres.
3. Motor end-plates on skeletal muscle, innervated by somatic motor nerves.
4. Certain synapses within the CNS including the Renshaw cells in the spinal cord.

nAChR and mAChR are widely distributed throughout the nervous system. In general, muscarinic synapses are found mainly in smooth and cardiac muscle and brain. Different sub-classes of mAChR have been defined pharmacologically (reviewed by Birdsall and Hulme, 1985) although all mAChR are activated by ACh and the muscarinic agonist oxotremorine and are inhibited by the muscarinic antagonist atropine.

1.1.4 The pharmacology of peripheral nAChR

Nicotinic synapses are found mainly at the neuromuscular junction and in autonomic ganglia. The pharmacology of the receptor at these two locations is different. In 1951, Paton and Zaimis reported that nAChR at the neuromuscular junction and the ganglia can be classified on the basis of antagonist selectivity to *bis*-trimethylammonium salts, $(CH_3)_3N^+ - (CH_2)_n - N^+(CH_3)_3$. Receptors in ganglia show a peak antagonist action at n = 5 or 6, whereas

receptors in the muscle show a peak when n = 10. This led to the classification of ganglionic receptors as "C_6" (sensitive to hexamethonium) and neuromuscular receptors as "C_{10}" (sensitive to decamethonium; Paton and Zaimis, 1951). Apart from this very general classification there are also several other differences in the pharmacology of the two receptors (reviewed by Koelle, 1975; Brown, 1980; and Taylor, 1985a,b). However, most nicotinic compounds act at both C_6 and C_{10} nAChR, making the classification less well defined (see below).

Compounds which act at the nAChR may be divided into two groups.

I, Agonists - compounds which bind at the agonist binding site and activate the receptor.

II, Antagonists - compounds which prevent the action of agonists.

I. Agonists

Nicotine, ACh and choline esters are all agonists of peripheral C_6 and C_{10} receptors. Both C_6 and C_{10} receptors are stereoselective with respect to nicotine, the naturally occurring isomer (-)-nicotine being more potent than (+)-nicotine (Barlow and Hamilton, 1965).

There are also several agonists specific for C_6 nAChR ; these include, 1,1-dimethyl-4-phenylpiperazinium (DMPP), lobeline, cytisine and coniine. DMPP is most commonly used as it is about three times more potent than nicotine (Taylor, 1985a).

The classification of agonists is complicated by the fact that in high doses ACh and nicotine block the action of the receptor (Taylor, 1985b) and in this respect ACh and nicotine behave as nicotinic antagonists. DMPP differs from nicotine in that much larger doses are required before blockade occurs (Taylor, 1985a). The depolarising agent decamethonium (originally used to characterise neuromuscular receptors) has also been shown to act as a nicotinic agonist (e.g. Gardner *et al.*, 1984).

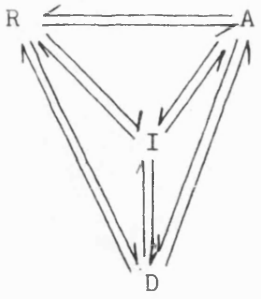
Electrophysiological studies have been carried out mainly at the neuromuscular junction to study the opening and closing of the nAChR-ionophore in response to agonists, and hence the functioning of the receptor. When an agonist binds, the receptor is activated and the channel opens (Adams, 1975). However, prolonged exposure of the receptor to the agonist leads to receptor desensitisation (Katz and Thesleff, 1957) which results in the closing of the channel and an increase in the receptor's affinity for agonists (Weber, 1975). To explain these results a three state model of the receptor was proposed, each state possibly representing a different conformational state of the receptor (reviewed by Changeux, 1981).

Three state model:

1. R, a resting state (channel closed, affinity for agonists low).
2. A, an activated state (channel open, affinity unknown)
3. D, a desensitised state (channel closed, affinity for agonist high).

However, more detailed studies have shown the need for at least a four state model of the receptor in which resting, open, fast-onset desensitised and slow-onset desensitised states are defined

(reviewed by Changeux, 1984; Karlin *et al.*, 1986).



R = resting state.

A = active state.

I = rapidly desensitised state

D = slowly desensitised state

The effect of noncompetitive blockers (see below) on ion flux and binding kinetics has provided further support for this allosteric four state model (Changeux, 1984).

II. Antagonists

Nicotinic antagonists may be divided into a) those which are non-depolarising blockers and b) those which are depolarising blockers.

a) Non-depolarising blockers

This group of compounds may be further subdivided into:

i) Competitive inhibitors: compounds which compete with the agonist for the binding site and hence prevent the opening of the ion channel.

ii) Noncompetitive inhibitors: compounds which prevent the activation of the nAChR but do not bind at the agonist binding site. These compounds inhibit the functioning of the receptor by one or more of three mechanisms:

1) stabilisation of the resting state;

Table 1.1. Summary of the classification of some nicotinic agents.

Type of Compound	Type of peripheral nAChR	
	Neuromuscular (C_{10})	Ganglionic (C_6)
I. AGONISTS	ACh Nicotine Choline esters (Decamethonium)	ACh Nicotine Choline esters DMPP Cytisine Lobeline Coniine
II. ANTAGONISTS		
a) Non-depolarising		
i) Competitive	α-bungarotoxin (α-BGT) d-tubocurarine dihydro-β-erythroidine (DHβE)	 Neosurugatoxin (NSTX) DHβE
ii) Noncompetitive	e.g. General and local anaesthetics Histrionicotoxin (HTX) Phencyclidine non-ionic detergents Chlorpromazine Aliphatic alcohols	Hexamethonium Mecamylamine Pempidine Chlorisondamine
b) Depolarising	Decamethonium ACh Nicotine	ACh Nicotine

2) blockade of the open channel;

3) promotion of desensitisation.

(Changeux *et al.*, 1984; Boyd and Cohen, 1984).

i) Competitive inhibitors

At the neuromuscular junction there are two well characterised competitive inhibitors, the α-neurotoxins and d-tubocurarine.

α-Neurotoxins: These are small polypeptides first purified by Chang and Lee (1962) from the venom of the elapid and hydrophobid snakes. An example is α-bungarotoxin (α-BGT) from *Bungarus multicinctus* which binds with high affinity to nAChR at the neuromuscular junction and hence causes paralysis of the victim. The binding of some α-neurotoxins (e.g. from *Naja naja siamensis* venom) is readily reversible whereas the reversibility of α-BGT takes several days (Lee, 1972, 1979). These compounds have proved to be extremely useful in the identification and purification of nAChRs (as will be discussed later).

The failure of α-BGT to block C_6 receptors has been reported several times (see Brown, 1979). Recently, attention has been focused on Kappa-bungarotoxin (κ-BGT), a neurotoxin isolated from the same venom as α-BGT (Grant and Chiapinelli, 1985). Unlike α-BGT, κ-BGT has been shown to bind with moderate affinity to both C_6 and C_{10} receptors (Grant and Chiapinelli, 1985).

d-Tubocurarine: Curare is a generic name for various South American arrow poisons. d-Tubocurarine is one of the pharmacologically active alkaloids which has been purified from curare and is a reversible competitive inhibitor at C_{10} receptors

(see Koelle, 1975). d-Tubocurarine has also been shown to be an antagonist at C_6 receptors (Ascher *et al.*, 1979).

Dihydro-β-erythroidine (DHβE): DHβE is a potent derivative of the alkaloid β-erythroidine. At the neuromuscular junction DHβE is a competitive antagonist resembling d-tubocurarine. However, although originally classified as a neuromuscular blocking antagonist it has also been shown to be an antagonist of equal potency at cat ganglia (Megirian *et al.*, 1955).

At ganglia, there is only one true competitive antagonist, neosurugatoxin.

Neosurugatoxin (NSTX): NSTX is a toxin isolated from the Japanese ivory shell *Babylonica japonica* (Kosuge *et al.*, 1981; 1982). A different compound called surugatoxin has been previously described (Kosuge *et al.*, 1972) although the potency of surugatoxin was later found to be a result of contamination by neosurugatoxin (Kosuge *et al.*, 1982). NSTX is highly specific for C_6 receptors and is ineffective at the neuromuscular junction (Hayashi and Yamada, 1975).

ii) Noncompetitive inhibitors

Classification of most non-depolarising antagonists is difficult, because many show both competitive and noncompetitive inhibition (see Brown, 1980). More recent studies have also shown that some 'competitive' ganglionic antagonists (e.g. hexamethonium)

may act at the ion channel rather than the agonist binding site (Rang, 1982; Gurney and Rang, 1984). The ganglionic antagonists (hexamethonium, mecamylamine and pempidine) have therefore been classified as noncompetitive inhibitors in Table 1.1.

A range of compounds has been shown to act as noncompetitive inhibitors at C_{10} receptors (see Spivak and Albuquerque, 1982). Some of these compounds are listed in Table 1.1. In the studies reported in this thesis two of these noncompetitive inhibitors were used; perhydrohistrionicotoxin (H_{12}HTX) and ketamine. Histrionicotoxin (HTX) is a spiropiperidine alkaloid originally isolated from the skin of the Columbian frog *Dendrobates histrionicus* (Daly *et al.*, 1971). It acts as a noncompetitive inhibitor by blocking the ion channel of the nAChR. HTX has been shown to be effective at nAChR at the neuromuscular junction (Albuquerque *et al.*, 1973) and at those on adrenal medullary cells (Kilpatrick *et al.*, 1981). The synthetic derivative H_{12}HTX is of equal potency as HTX (Spivak *et al.*, 1982). The anaesthetic ketamine has also been shown to interact with the ion channel of the peripheral C_{10} receptor (Maleque *et al.*, 1981).

b) Depolarising blockers; e.g. Decamethonium

Decamethonium is a depolarising agent used in the original classification of C_{10} receptors. However, it has also been shown to act as an agonist (e.g. Gardner *et al.*, 1984) and causes desensitisation (see Grundy, 1985). There is also evidence that additionally decamethonium acts at ganglionic receptors by blocking the channel opened by cholinergic agonists (Ascher *et al.*, 1979).

The pharmacological specificity of the nAChR has therefore been extensively studied. Electrophysiological studies have also provided information about the release of ACh at the neuromuscular junction and the functioning of the nAChR on postsynaptic membranes. In contrast, our understanding of the biochemistry of the nAChR has only been developed over the last 15 years. The biochemist has taken advantage of the cholinergic system present in the electric fish *(Torpedo* genus) and electric eel *(Electrophorus electricus)*. Present within these organisms are electric organs consisting of stacks of flattened, multinucleated cells arranged in prisms. The cells are known as electroplaques or electroplax and are phylogenetically derived from muscle cells (reviewed by Aronstam, 1982). All synapses within electric organs respond to ACh and have a purely nicotinic pharmacology which means that they are a rich source of nAChR.

With the use of the specific probes the α-neurotoxins, purification of the nAChR has been possible. As a result the nAChR is the most widely studied and best understood neurotransmitter receptor (recently reviewed by Changeux, 1984; Dolly and Barnard, 1984; McCarthy *et al.*, 1986; and Hucho *et al.*, 1986).

The next section summarises the present knowledge of the nAChR from *Torpedo*.

1.2 THE nAChR

1.2.1 A pentameric transmembrane protein

The nAChR from fish electroplaques has been isolated from detergent extracts using affinity chromatography with purified snake-venom neurotoxins as affinity ligands (Olsen *et al.*, 1972; Schmidt and Raftery, 1972; Klett *et al.*, 1973). The total molecular weight of the receptor isolated from *Torpedo* is about 250,000. It is composed of four different subunits with apparent molecular weights of 40,000, 50,000, 60,000 and 65,000, designated α, β, γ, δ respectively. Each subunit is a glycoprotein containing 4 - 7% carbohydrate (Raftery *et al.*, 1976) and the four different subunits are assembled to form a pentamer, $\alpha_2\beta\gamma\delta$ (Reynolds and Karlin, 1978; Lindstrom *et al.*, 1979; Raftery *et al.*, 1980). Associated with these five polypeptides is a peripheral protein of molecular weight 43,000 on the cytoplasmic surface of the membrane (Sobel *et al.*, 1977). This protein has been assigned a structural role (Barrantes *et al.*, 1980) and possibly acts as a link between the membrane containing the receptor and the cytoskeleton (Porter and Froehner, 1983; Burden *et al.*, 1983; Walker *et al.*, 1984a). The nAChR from *Torpedo* has been shown by sucrose density centrifugation to exist in two forms, a heavy (dimer) form and a light (monomer) form (Chang and Bock, 1977; Reynolds and Karlin, 1978). The dimer results from the formation of a disulphide bond between the δ-subunits of two single receptors (Sobel *et al.*, 1977; Suarez-Isla and Hucho, 1977). The nAChR isolated from mammalian muscle is of similar size to the *Torpedo* receptor but no dimeric forms have been observed.

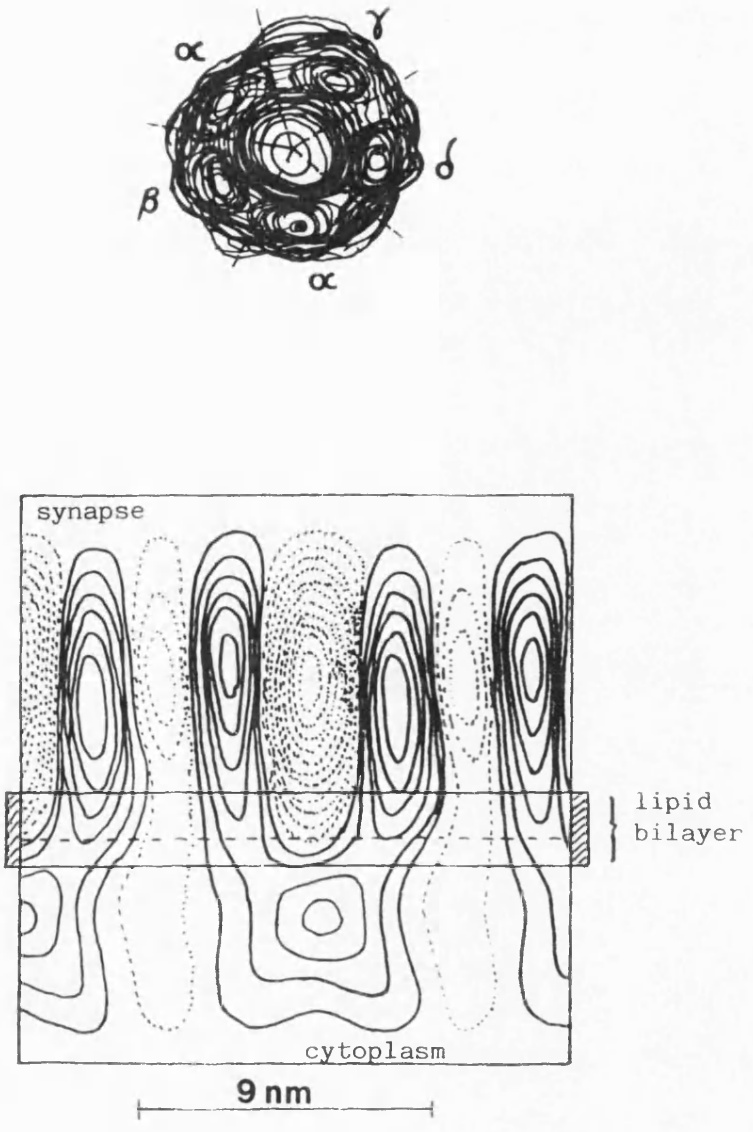


Fig. 1.2. Quaternary configuration of the nAChR, determined by 3-D electron image analysis of tubular crystals grown from native *Torpedo marmorata* membrane vesicles (from Brisson and Unwin, 1985).

a) Receptor viewed from the top (looking from extracellular side). The receptor consists of 5 subunits (regions indicated by the dotted lines) equally spaced around a central pore thought to be the ion channel.

b) Side view (through the channel axis) of the receptor. The central plane of the bilayer is indicated by the broken line.

From X-ray diffraction, electron microscope staining studies and immunological studies (Ross *et al.*, 1977; Klymkowsky and Stroud, 1979; Tarrab-Hazdai *et al.*, 1978; Strader *et al.*, 1979) the *Torpedo* nAChR has been shown to be a transmembrane protein with its polypeptides arranged in a circle around a central pore. The work of Brisson and Unwin (1985) has provided the most recent picture of the quaternary structure of the receptor (Fig. 1.2). From these studies the receptor is seen to be highly symmetrical around the axis of the presumed pore, the protein extending above and below the lipid bilayer.

1.2.2 The primary structure of the subunits

Once the subunits of the *Torpedo* nAChR had been isolated attempts were made to sequence them (Devillers-Thiery *et al.*, 1979; Raftery *et al.*, 1980). Total (100%) homology was found between the first twenty amino acids at the N-terminus of the α-chain from *Torpedo marmorata* and *Torpedo californica*. Homology (35 - 50%) was also shown between all four subunits in this region (Raftery *et al.*, 1980). However, complete sequences of the nAChR subunits were not determined using this method, instead the primary sequences were obtained using recombinant DNA techniques. A short stretch of known N-terminal amino acid sequence was used to construct an equivalent DNA fragment so that it could be used as a ^{32}P-labelled probe to screen a cDNA library prepared from *Torpedo* electroplaques. Using similar techniques the cDNA sequences coding for the respective precursors of the α, β, γ and δ polypeptide chains of *Torpedo californica* have been determined (Noda, 1982; 1983a; 1983b; Claudio *et al.*, 1983). The α-chain precursor of nAChR

from *Torpedo marmorata* has also been sequenced (Devillers-Thiery *et al.*, 1983; Sumikawa *et al.*, 1982).

The sequences of each chain show a high degree of homology (~ 40%). The α and β chains being more similar to each other than to γ and δ which in turn show more homology to each other than to α or β. These results suggest that the genes for the four polypeptide chains may have originated from a pair of (and initially one) common ancestral genes (Raftery *et al.*, 1980).

Once *Torpedo* nAChR subunit cDNA was available, it was used to probe libraries prepared from human (Noda *et al.*, 1983c), bovine (Noda *et al.*, 1983c), chicken (Ballivet *et al.*, 1982) and mouse muscle cell lines (Merlie *et al.*, 1984).

Comparison of the α-subunit sequences from different species has shown that the AChR is highly conserved throughout evolution. Noda *et al.* (1983c) showed 97% homology between α-chains of human and calf receptor and 80 - 81% homology between human and *Torpedo* α-chains. It was proposed that the α-subunit has evolved more slowly than the other subunits and that the pentameric structure $\alpha_2\beta\gamma\delta$ was established 550 - 690 million years ago (Kubo *et al.*, 1985).

Using similar techniques a fifth polypeptide chain called ε has recently been isolated from calf muscle (Takai *et al.*, 1985). The actual role of this ε-subunit is not known but it has been suggested that it may replace the γ-subunit at certain stages of muscle development (Takai *et al.*, 1985).

1.2.3 Post-translational modifications

In *Torpedo* electroplaques, approximately 2.4% of the total messenger RNA (mRNA) codes for the nAChR (Mendez *et al.*, 1980). As

mentioned above, translation of this mRNA results in precursors of each subunit. These precursors contain a leader sequence of 17 - 24 amino acids which are mainly hydrophobic and are absent in the mature subunit (Anderson and Blobel, 1981), suggesting that post-translational modification occurs. The requirement of cellular components for the biosynthesis of functional nAChR was demonstrated by the translation of mRNA isolated from *Torpedo* in either a cell-free system (Mendez *et al.*, 1980; Anderson and Blobel, 1981; Sumikawa *et al.*, 1981) or a *Xenopus* oocyte system (Sumikawa *et al.*, 1981; Barnard *et al.*, 1982; Mishina *et al.*, 1984). In the cell-free systems, although the individual subunits for the receptors were identified,no receptor activity was observed, as shown by the lack of [^{125}I] α-BGT binding (Anderson and Blobel, 1981; Sumikawa *et al.*, 1981). In contrast, when mRNA (either isolated from cells or synthesised *in vitro* using a cDNA template) was injected into oocytes, nAChR was synthesised, post-translational processing was carried out and functional receptors were inserted in the oocyte membrane (Sumikawa *et al.*, 1981). Following translation the native polypeptides are further processed (see Hucho, 1986). The four subunits are glycosylated (Mattson and Heilbronn, 1975; Raftery *et al.*, 1976) and recent studies using site-directed mutagenesis (Mishina *et al.*, 1985) have suggested that the N-glycosylation site at asparagine residue 141 on the α-subunit is important for the assembly and function of the receptor. There is also evidence that all four subunits are phosphorylated (Vandlen *et al.*, 1979), covalently linked to fatty acids (Olson *et al.*, 1984) and methylated (Flynn *et al.*, 1982;

Nuske, 1986). However, the exact roles of these modifications relating to receptor function have yet to be defined.

1.2.4 The ACh binding site

Although the four subunits show a large degree of sequence homology, they are not identical. It is only the α-subunits which carry a high affinity binding site for ACh, d-tubocurarine and α-BGT (Karlin *et al.*, 1986).

Identification of the ACh binding site was carried out using the sulphydryl affinity labelling reagents bromoacetylcholine (Br-ACh; Silman and Karlin, 1969) and 4-(N-maleimido)-benzyl trimethylammonium iodide (MBTA; Reiter *et al.*, 1972). These ligands bind covalently to SH groups at or near to the ACh site after reduction of sulphide bonds with dithiothreitol and hence prevent the binding of α-BGT. However, the two α-subunits are not equivalent, one site on the receptor is easily labelled with the affinity ligands (Dalmle *et al.*, 1978; Karlin, 1980; Wolosin *et al.*, 1980) but high concentrations of affinity ligands and reducing agents are required to label the second site (Wolosin *et al.*, 1980; Walker *et al.*, 1984b). Kinetic binding studies have shown that α-BGT binds equally to each subunit (Lee, 1979) whereas d-tubocurarine shows a 300 fold difference in affinity for the two sites (Neubig and Cohen, 1979). This may be a result of differences in the post-translational modifications of the two subunits, alternatively, it may be a reflection of the different environments of the subunits within the receptor complex (Ratnam *et al.*, 1986). Indeed recent evidence (Ratnam *et al.*, 1986) has shown that one of the α-subunits is more glycosylated than the

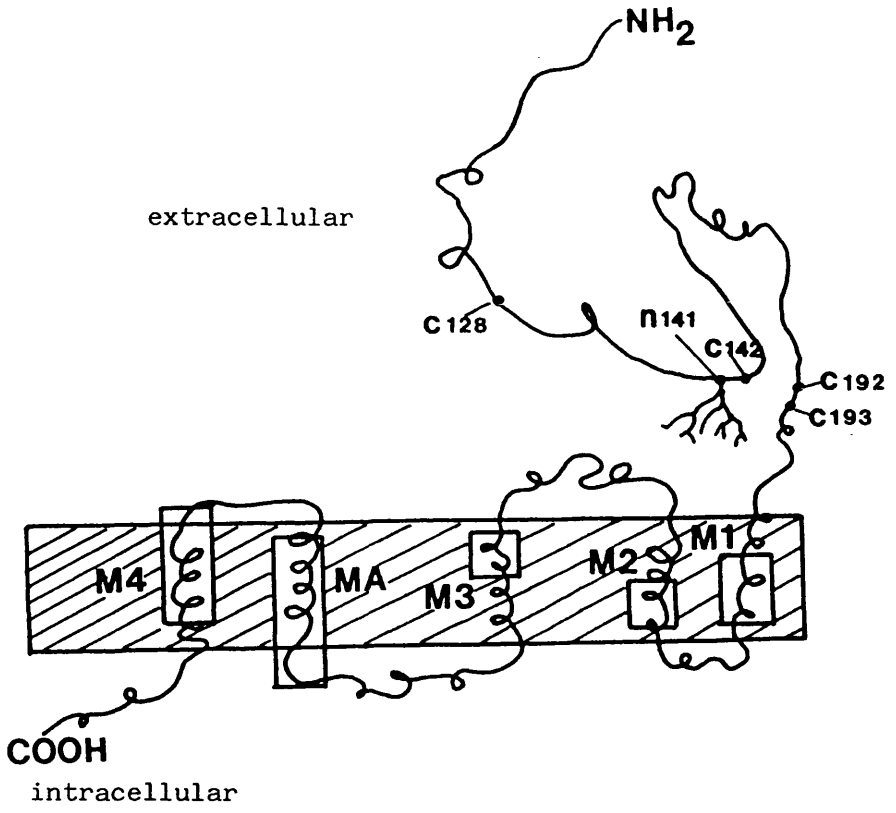


Fig. 1.3. Model of the predicted transmembrane organisation of the α-subunit of the nAChR (Guy, 1984; Finer-Moore and Stroud,1984). Counting from the N-terminus the membrane spanning regions were named M1, M2, M3, MA (or M5) and M4. The regions M1, 2, 3, 4 are hydrophobic whereas MA is amphipathic (hydrophobic groups along one face and hydrophilic groups along the other face of the helix).

Recent evidence (Mishina *et al.*, 1985) has shown that an N-glycosylation site (n141) is important for the assembly and the function of the receptor.

In the α-subunit, the ACh binding site has been shown to be close to cysteine(192) which forms a disulphide bond with cysteine (193) (Kao *et al.*, 1984).

other; the one which is least glycosylated showing higher affinity for d-tubocurarine.

From analysis of the amino acid sequence of the α-subunit Numa *et al.*, (1984) proposed that the important disulphide bond present in the ACh binding site was the bridge formed between cys 128 and cys 142. After this suggestion various models of the ACh binding site were proposed (e.g. White, 1985). However, studies by Kao *et al.*, (1984) revealed that it is the disulphide bond between the adjacent cysteine residues 192 and 193 which binds the affinity ligand MBTA. Similar experiments have shown that α-BGT and d-tubocurarine bind to fragments of the α-subunit which contain the cys 192 and cys 193 residues (Wilson *et al.*, 1985; Neumann *et al.*, 1986). There is therefore increasing evidence that the N-terminal region containing cys 192 and cys 193 is the area to which ACh and competitive antagonists bind (see Fig. 1.3).

1.2.5 Models of the tertiary structure

The exact tertiary structure of the nAChR is still unknown because crystallisation and X-ray diffraction analysis of the receptor has not yet been possible. However, from the biochemical and biophysical information obtained so far, predictions of the arrangement of the α-polypeptide chain have been made (Noda *et al.*, 1983c; Claudio *et al.*, 1983; Devillers-Thiery *et al.*, 1983; Finer-Moore and Stroud, 1984; Gury, 1984).

Using Fourier and hydrophobicity analysis, Finer-Moore and Stroud (1984), and Guy (1984) predicted that each subunit contained four hydrophobic (M1 - M4) and one amphipathic (MA) transmembrane

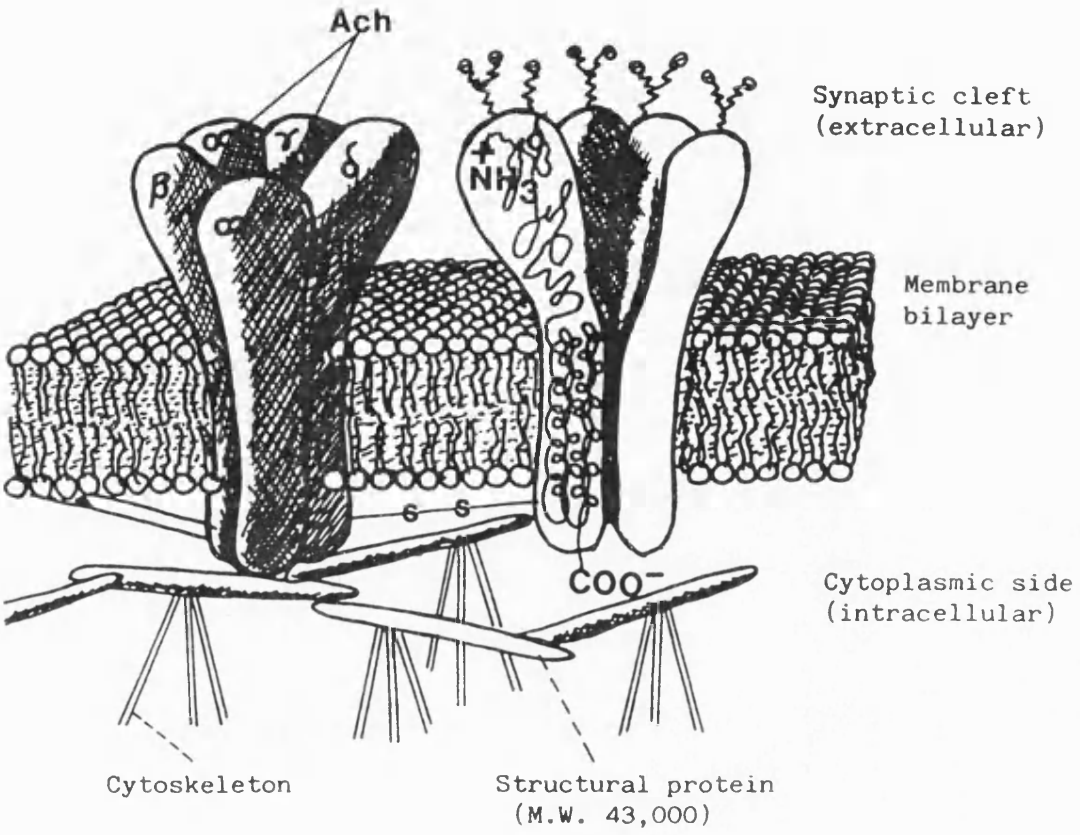


Fig. 1.4. 3-D model of the nAChR from *Torpedo californica* (from Lindstrom *et al.*, 1984; Hucho, 1986).

The five polypeptide chains ($\alpha_2\beta\ \gamma\ \delta$) span the membrane, each bearing oligosaccharide chains on the outer surface (zig-zag lines). However, the exact subunit arrangement is unknown (either the β or γ subunit lies between the two α subunits). In *Torpedo* the receptor occurs in the light form ($\alpha_2\beta\ \gamma\ \delta$) or as a heavy-form dimer resulting from the association of two light forms by δ - δ disulphide bridge. The receptors are cross-linked to membrane structural proteins (M.W. 43,000) which may link the receptor to the cytoskeleton.

The N-terminus of each polypeptide is on the extracellular surface and the C-terminus is probably intracellular, the polypeptide chain passing the membrane five times (see Fig. 1.3).

helices (Fig. 1.3). These workers suggested that the MA region forms the wall of the proposed ion channel.

Monoclonal antibodies raised to specific domains of the receptor subunits have been used to verify the proposed secondary structure. Using such techniques Lindstrom *et al.* (1984) and Young *et al.*, (1985) showed that the C-terminus of the subunits was on the cytoplasmic side of the membrane.

However, although the model of Finer-Moore and Stroud and also Guy is widely accepted, the arrangement of the polypeptide chains and the number of membrane spanning regions is still controversial; recent immunological data suggest five (Young *et al.*, 1985) and even seven (Neumann *et al.*, 1984; Criado *et al.*, 1985) membrane spanning domains.

Other structural predictions have been the number and location of asparagine-linked glycosylation sites and the location of cys-cys disulphide bonds near the ACh binding site (Fig. 1.3). Using all the information available, a 3-D model of the nAChR complex in the membrane bilayer has been proposed (see Fig. 1.4).

1.2.6 Biochemical evidence for a nAChR ionophore

Evidence for the ionophore being an integral part of the pentameric $\alpha_2\beta\ \gamma\ \delta$ structure was obtained by experiments in which the purified AChR was reconstituted into vesicles (reviewed by McNamee and Ochoa, 1982; Anholt *et al.*, 1983). The function of the receptor can be assayed by either measuring agonist-induced influx of $^{22}Na^+$ into the vesicles, or by measuring the opening and closing of the channels in individual receptors using patch-clamp techniques (Suarez-Isla *et al.*, 1983).

Further evidence for an AChR ionophore was provided by the expression of functional receptor from cloned cDNAs in *Xenopus* oocytes (Mishina *et al.*, 1984). Comparison of the channel activities of *Torpedo* and calf receptors (expressed on the surface of oocytes) showed that the response is species specific; the amplitude is the same but the response is longer for the calf nAChR (Sakmann *et al.*, 1985). By taking α, β, γ subunit specific mRNA from *Torpedo* and δ-subunit specific mRNA from calf to form hybrid mRNA, Sakmann and co-workers showed that the resulting channel properties of the expressed receptor were characteristic of the calf type receptor. These results suggest that the δ subunit plays a specific role in the gating of the ion channel.

These results, together with the predicted folding of the receptor polypeptides within the plasma membrane (see Figs. 1.3 and 1.4) and the 3-D electron density image of the receptor (see Fig. 1.2) provide substantial evidence for the ionophore being an integral part of the receptor.

1.2.7 The binding sites for noncompetititve antagonists

The sites to which noncompetitive antagonists bind are still unknown and may be as diverse as their structures. A single high affinity binding site for noncompetitive antagonists per nAChR molecule has been suggested which is characterised by its sensitivity to HTX and may be related to the ion-channel (Heidmann *et al.*, 1983; Oswald *et al.*, 1983; Haring and Kloog, 1984). There are also low affinity binding sites for noncompetitive antagonists which are insensitive to HTX and possibly located away from the channel at the protein-lipid interface (Heidmann *et al.*,

1983). To gain more information about the binding sites for non-competitive antagonists and the gating mechanism of the receptor ionophore, binding experiments have been carried out using photo-affinity labelling. Using this technique the azido derivative of the local anaesthetic trimethisoquin was shown to preferentially bind to the δ subunit of the receptor (Saitoh *et al.*, 1980). Later studies using unmodified noncompetitive antagonists as photo-affinity labels showed labelling of α, β and δ subunits and using [^{3}H]triphenylmethylphosphonium ([^{3}H]TPMP$^+$) the binding was shown to be in the M2 region of the proposed arrangement of the poly-peptide chain (see Fig. 1.3; Hucho, 1986). This area is not in the proposed channel-forming MA segment.

Hucho (1986) suggests that the region labelled by [^{3}H]TPMP$^+$ may form part of the ion-channel which is consistent with the work of Taylor who has shown that noncompetitive antagonists bind at a site 4.8 nm away from the agonist binding site (see Fig. 1.2; Colquhoun, 1986).

1.2.8 Comparison of the nAChR from *Torpedo* and vertebrate muscle

Pharmacologically, the nAChR isolated from electric fish and vertebrate muscle show no major differences, they are both excited by ACh and specifically blocked by α-BGT (Changeux, 1981). Electro-physiological studies have also shown that the two types of recep-tors are similar (Peper *et al.*, 1982). The structure of the *Torpedo* nAChR has also been shown to be very similar to the receptor from vertebrate muscle using immunological techniques. Antisera and monoclonal antibodies raised against *Torpedo* nAChR cross react with antigenic determinants corresponding to the α, β,

and δ chains in rat, bovine and human muscle (Lindstrom *et al.*, 1978, 1979). The primary structures and molecular masses of the subunits are also similar but not identical (Kubo *et al.*, 1985; Einiarson *et al.*, 1982). The observed similarities between the two receptors is not surprising because electroplaque cells are evolved from embryonic striated muscle cells.

1.3 EVIDENCE FOR NEURONAL nAChR

In 1934, Dale proposed that ACh might act as a transmitter in the CNS, although there was no direct evidence. This was provided soon after by the identification of ACh and acetylcholinesterase in various regions of the CNS (see Krnjević, 1975).

1.3.1 Electrophysiological studies

The existence of nAChR in the mammalian CNS has since been demonstrated by measurement of the excitatory responses of neurones following iontophoresis of ACh or nicotinic agonists (see Krnjević, 1975; Phillis, 1976). The excitatory action of nicotine has been recorded in most regions of the CNS but it is the Renshaw cell (in the spinal cord) which has often been the choice of study because it is a large and easily identifiable neurone. The characteristic feature of the nicotinic response is a quick excitation which is rapidly reversible and is prevented by ganglionic blocking agents (Krnjević, 1975). It was also found that d-tubocurarine and related compounds were not useful nicotinic antagonists in the CNS because they excite many neurons.

More recent electrophysiological evidence for central nAChR displaying ganglionic pharmacology has been provided by McLennan and Hicks (1975), Lichtensteiger *et al.* (1982) and Clarke *et al.* (1985b).

1.3.2 The use of α-BGT as a probe for neuronal nAChR

α-BGT has been an invaluable probe for the study of the nAChR from skeletal muscle and *Torpedo* electric organ. In the search for a central nAChR, α-BGT was therefore initially used as a

ligand. However, the suitability of α-BGT as a probe for a neuronal nAChR has been questioned (Oswald and Freeman, 1981).

Electrophysiological studies showed that α-BGT can block cholinergic retinotectal transmission in the toad (Freeman, 1977), goldfish (Freeman *et al.*, 1980) and frog sympathetic neurones (Marshall, 1981). In contrast, α-BGT had no effect on transmission in rat retinotract systems (Schmidt and Freeman, 1980), rat cervical ganglion (Brown and Fumagalli, 1977) or chick ciliary ganglion (Carbonetto *et al.*, 1978; Kouvelas *et al.*, 1979; Ravin and Berg, 1979). α-BGT also failed to block synaptic transmission at nicotinic cholinergic synapses in the frog spinal cord (Miledi and Szczepaniak, 1975) and in Renshaw cells of the cat spinal cord (Duggan *et al.*, 1976).

However, [^{125}I]α-BGT binds with high affinity to rat brain membranes (Schmidt *et al.*, 1980) and the binding is saturable and displaced by nicotinic agonists, but unlike the binding to *Torpedo* or muscle nAChR it is more readily reversible (Schmidt *et al.*, 1980). The physiological relevance of α-BGT binding sites has been questioned because it was found that some neurones contain both α-BGT binding sites that are not functional nAChR,and,nAChR that are activated by ACh but do not bind α-BGT (Patrick and Stallcup, 1977; Jacob and Berg, 1983). [^{125}I]α-BGT has also been shown to bind to structures which lack any cholinergic innervation (Hunt and Schmidt, 1979), and to bind to extrasynaptic structures (Ninkovic and Hunt, 1983; Loring *et al.*, 1985).

1.3.3 Purification of an α-BGT binding component

Purification of an α-BGT binding protein from rat brain has

been difficult because of the low amount of binding sites (1.4 - 6 pmoles/g wet wt; see Schmidt *et al.*, 1980) compared with the relatively high amounts obtained from electric organs (e.g. from *Torpedo marmorata* 1720 pmoles [^{125}I]α-BGT sites/g wet wt; Eldefrawi and Eldefrawi, 1973). Nevertheless, using α-BGT as an affinity ligand early studies reported the purification of α-BGT binding components from goldfish brain (Oswald and Freeman, 1979), chick retina (Betz, 1981), chick optic lobe (Norman *et al.*, 1982), mouse brain (Seto *et al.*, 1981) and rat brain (Wonnacott *et al.*, 1982). In common with the peripheral nAChR, the brain α-BGT binding proteins are glycoproteins, they are soluble in non-ionic detergents and there is indirect evidence that they bind the affinity alkylating agents MBTA and Br-ACh (Lukas and Bennett, 1980). These properties were initially taken as confirmation of their identity as the central nAChR. However, there were some major differences such as the sedimentation coefficient, the isoelectric point, subunit composition and the affinity for α-BGT (see Wonnacott *et al.*, 1982). The α-BGT binding proteins were shown to be composed of one type of subunit (Seto *et al.*, 1980; Norman *et al.*, 1982; Betz *et al.*, 1982). However, more recently Conti-Tronconi *et al.* (1985) have reported the purification of an α-BGT binding protein from chick optic lobe which contained three subunits with apparent molecular weights 48,000, 56,000 and 69,000, although the stoichiometry is unknown. N-terminal amino-acid sequencing showed that the 48,000 molecular weight component was highly homologous with the α-subunit of electric organ and muscle receptors. Unfortunately,sequencing of the other subunits was not possible because of N-terminal blockage. However, the 58,000

molecular weight subunit was also shown to resemble the α-subunit of the peripheral type receptor by its ability to be labelled with Br-ACh and cross-linked to α-BTX. All the subunits cross reacted with monoclonal antibodies directed against chick muscle receptor.

Further evidence for the brain α-BGT binding protein being related, but not identical to nAChR at the neuromuscular junction, was provided in the same year by Kemp *et al.* (1985) who showed that the α-BGT binding protein from rat brain was composed of three subunits of apparent molecular weights of 49,000, 53,500 and 55,000. Two α-BGT binding sites were identified and it was the 55,000 molecular weight protein which bound the affinity ligand [^{3}H] MBTA.

These results are consistent with those of Conti-Tronconi *et al.* (1985) in that it is the subunit of molecular weight around 55,000 which has characteristics of the α-subunit from *Torpedo* or muscle nAChRs. This neuronal α-BGT binding subunit is also of a molecular weight similar to the single subunits originally isolated by Seto *et al.* (1980), Norman *et al.* (1982) and Betz *et al.* (1982). The purification of additional subunits by the more recent studies may be a result of improvements in receptor purification techniques and the use of protease inhibitors (Conti-Tronconi *et al.*, 1985).

To compare the neuronal α-BGT binding sites with nAChR isolated from *Torpedo* or muscle, several immunological studies have been carried out. Cross reactivity between antisera to nAChR from *Torpedo* with the α-BGT binding component of rat brain has been shown (Block and Billiar, 1979; Wonnacott *et al.*, 1982; Mills and Wonnacott, 1984). Antisera to receptor from denervated cat muscle

and two monoclonal antibodies to receptors from chick muscle have also been shown to cross react to an α-BGT binding protein from chick brain (Norman *et al.*, 1982; Mehraben *et al.*, 1984). However, there have also been reports of no interaction between the α-bungarotoxin binding component purified from rat brain and antisera against nAChR from *Torpedo* (Morley *et al.*, 1983).

1.3.4 Purification of other ligand binding proteins

Evidence for the existence of at least two distinct types of neuronal nAChR has recently been provided (Schneider *et al.*, 1985; Wonnacott, 1986; Whiting and Lindstrom, 1986). Using α-toxin affinity purification, high affinity [^{3}H] nicotine binding sites have been separated from α-BGT binding proteins in rat brain (Wonnacott, 1986), and by similar techniques high affinity [^{3}H] ACh binding sites have been purified from chick optic lobe which are distinct from the α-BGT binding proteins (Schneider *et al.*, 1985). Information about the subunit composition of the agonist binding components is not yet available although Whiting and Lindstrom (1986) have used monoclonal antibody affinity chromatography to purify a neuronal nAChR which is composed of two subunits, apparent molecular weights 48,000 and 59,000. This protein shows little structural homology to muscle nAChR but appears to be related to the high affinity [^{3}H] nicotine binding sites in brain. The binding of [^{3}H]nicotine is displaced by antibodies raised against the purified receptors and monoclonal antibodies to the purified receptor label brain sections with the same pattern as [^{3}H]nicotine (Whiting and Lindstrom, 1986).

In the recent search for neuronal nAChRs, studies have been carried out using PC-12 cells, a tumour cell line derived from rat adrenal medulla. These cells have α-BGT binding sites which are not functional nAChR (Hall, 1986). Using cloning techniques, a cDNA clone was obtained from PC-12 cells which codes for a protein that is related (47% homology) to,but different from, the α-subunit from muscle receptor (Boulter *et al.*, 1986). Evidence for the cDNA clone encoding a neuronal type receptor was obtained by hybridisation studies. The clone did not bind to mRNA in normal or denervated muscle but hybridised to specific brain areas in the rat and to bovine adrenal medulla (Boulter *et al.*, 1986). A large amount of labelling was also found in the area known as the medial habenula, an area to which nicotine and ACh,but not α-BGT bind (Clarke *et al.*, 1985a). These results suggest that the PC-12 clone encodes a subunit which is distinct from the muscle nAChR α-subunit. Although the muscle α-subunit and the PC-12 subunit are probably products of two separate genes there are a number of similarities such as the presence of the four extracellular cysteine residues and the four hydrophobic regions and one amphipathic region. In view of these similarities one might expect the functioning of the receptors to also be comparable.

1.3.5 Comparison of the binding of radiolabelled nicotinic ligands in rodent brain

The nicotinic ligands [^{125}I]α-BGT, [^{3}H]nicotine and [^{3}H]ACh have been shown to specifically bind to rodent brain (Clarke *et al.*, 1985a). Comparison of the regional distribution of binding of each ligand and the displacement of the binding by a range of

unlabelled cholinergic drugs has provided further information about central nAChRs. In view of the problems with α-BTX as a probe for neuronal nAChR (see above) more recent studies have used tritiated nicotine and acetylcholine of high specific activity to study central agonist binding sites.

a) [^{3}H]nicotine

Saturable binding of [^{3}H] nicotine to rodent brain has been demonstrated and derivation of kinetic constants have yielded K_d values ranging from 1 - 50 nM, consistent with a high affinity binding site (Yoshida and Imura, 1979; Romano and Goldstein, 1980; Marks and Collins, 1982; Costa and Murphy, 1983). There have also been reports of two [^{3}H]nicotine binding sites, one of high and one of low affinity (Romano and Goldstein, 1980; Shershen *et al.*, 1981; Yoshida *et al.*, 1982; Costa and Murphy, 1983; Sloan *et al.*, 1984; London *et al.*, 1985a), although the specificity of the low affinity site has been questioned because it is often only observed when binding is carried out at 4°C (Marks and Collins, 1982) and it is not observed in the presence of protease inhibitors (Lippiello and Fernandes, 1986).

The high affinity [^{3}H]nicotine binding site is stereoselective, displaying a preference for the naturally occurring (-)-isomer (see Wonnacott, 1986). Recent evidence (Sloan *et al.*, 1985; Abood *et al.*, 1985) suggests that the two enantiomers of nicotine may act preferentially at different sites. It has therefore been proposed (Sloan *et al.*, 1985) that (-)-[^{3}H]nicotine should be used in binding studies to avoid any spurious effects caused by the (+)-isomer.

High affinity [^{3}H]nicotine binding is displaced by cholinergic agonists (e.g. cytisine, DMPP and lobeline), but high concentrations (> μM) of the well established nicotinic antagonists (e.g. hexamethonium, mecamylamine) are required to displace the binding (Romana and Goldstein, 1980; Marks and Collins, 1982; Costa and Murphy, 1983). Although nicotine is able to displace [^{125}I]α-BGT binding, α-BGT is ineffective at displacing [^{3}H]nicotine binding (Marks and Collins, 1982; Rapier *et al.*, 1985). The failure of many nicotinic antagonists to compete for the high affinity [^{3}H]nicotine binding site may be a result of the artificial situation, where the receptors are exposed to agonists for long periods and the receptor is converted to a desensitised form (Clarke *et al.*, 1985a). There are however two antagonists which displace the high affinity [^{3}H] nicotine binding: NSTX and DHβE. The ganglionic antagonist NSTX is totally specific for the high affinity [^{3}H]nicotine site (Hayashi *et al.*, 1984; Rapier *et al.*, 1985; Yamada *et al.*, 1985) and does not inhibit [^{125}I]α-BGT binding (Rapier *et al.*, 1985). The other antagonist DHβE, is effective at displacing high affinity [^{3}H] nicotine binding (Clarke *et al.*, 1984; Wonnacott, 1987) as well as [^{125}I]α-BGT binding (Schmidt, 1977; Wonnacott, 1987).

b) [^{3}H]ACh

Characterisation of central nicotinic receptors has also been studied by measurement of [^{3}H]ACh binding to rat brain membranes in the presence of excess atropine to block muscarinic receptors (Schwartz *et al.*, 1982). [^{3}H]ACh binds to a single high affinity nicotinic receptor; the binding is displaced by cholinergic agonists such as nicotine and cytisine and displays

stereoselectivity for (-)-nicotine (Schwartz *et al.*, 1982). As observed for the [^{3}H]nicotine binding site, the [^{3}H]ACh recognition site shows low affinity for most nicotinic antagonists except DHβE (Schwartz and Keller, 1983).

More recently both high affinity and low affinity nicotinic binding of [^{3}H]ACh to rat brain membranes has been demonstrated (Reulecke and Hucho, 1985), consistent with the reported high and low affinity binding sites for [^{3}H]nicotine.

c) Other radiolabelled ligands

Williams and Robinson (1984) recently reported that [^{3}H] DHβE binds specifically to rat brain tissue and two binding sites are identifiable, one of high affinity and one of slightly lower affinity. The binding of this radiolabelled antagonist was displaced by nicotinic agonists but as in the case of [^{125}I]α-BGT, [^{3}H]nicotine and [^{3}H]ACh binding, nicotinic ganglionic antagonists (mecamylamine, hexamethonium, pempidine) were ineffective at displacing bound ligand. It was concluded that [^{3}H]DHβE bound to a nicotinic recognition site in the brain which was neuromuscular rather than ganglionic in nature. This interpretation may be naive particularly in the light of the reported equipotency of DHβE at cat ganglia and muscle (Megirian *et al.*, 1955).

[^{3}H]Tubocurarine has been shown to specifically bind to rat brain membranes (Nordberg and Larsson, 1980) and displays similar binding constants and regional distribution binding as [^{3}H]nicotine (Larsson and Nordberg, 1985). However, the use of other radiolabelled antagonists to label central nicotinic sites has not yet been possible.

d) Comparison of the regional distribution of radiolabelled ligand binding

Although [^{3}H]nicotine, [^{3}H]ACh and [^{125}I]α-BGT bind to many brain areas,a lack of correlation between the distribution of agonist and α-BGT binding sites has been demonstrated (Marks and Collins, 1982; Schwartz *et al.*, 1982; Clarke *et al.*, 1985a).There is therefore substantial evidence suggesting that α-BGT and nicotinic agonists do not necessarily label the same molecules. This conclusion is supported by the recent purification of two distinct proteins from rat brain (Wonnacott, 1986), one binding [^{125}I]α-BGT and one which binds [^{3}H]nicotine with high affinity. The evidence to date is therefore in favour of there being two distinct agonist binding sites in rat brain and that α-BGT binds to only one site which is possibly the low affinity [^{3}H]nicotine binding site (Wonnacott, 1986).

The binding of radiolabelled nicotinic ligands to rat brain membranes provides evidence for the presence of central nAChR. However, these studies give no information about the functioning of the receptors. Alternative studies have therefore been carried out to study the action of nicotine in the CNS.

1.3.6 The action of nicotine in the CNS

Many of the early side effects observed by people beginning to smoke such as vomiting and irritability, may be attributed to the central actions of nicotine(Ashton and Stepney, 1983). These effects have been studied in the laboratory by the intravenous injection of nicotine into experimental animals. Early studies using [^{14}C] nicotine showed that high levels of nicotine rapidly

appeared in the brain (reviewed by Aceto and Martin, 1982). Nicotine stimulates all areas of the CNS producing increased levels of behavioural activity and may produce tremors and convulsions (Caulfield and Higgins, 1983; Nordberg and Sundwall, 1983). It is also capable of stimulating the vomiting and respiratory centres of the brainstem (Beleslin *et al.*, 1983) and stimulates the hypothalamus to release antidiuretic hormone resulting in the retention of fluid (Volle and Koelle, 1975). Other central effects include the improvement of learning and memory processes (Nordberg and Bergh, 1985) and the release of a range of neurotransmitters (reviewed by Aceto and Martin, 1982; Balfour, 1982).

The action of nicotine in the CNS is typically blocked by ganglionic antagonists (e.g. mecamylamine) which also penetrate centrally (Clarke *et al.*, 1985). There is therefore evidence for the presence of nAChR within the CNS with characteristics similar to the C_6 nAChR. However, the functional significance of the central nAChR is not well understood.

The neurotransmitter releasing action of nicotine provides a system for the study of a 'functional' nAChR. Early studies by Westfall (1974a) showed that nicotine could evoke the release of noradrenaline from hypothalamic slices and dopamine (DA) from striatal brain slices, suggesting that nicotine had a direct effect on the nerve terminal.

The next section describes the evidence for the nicotinic regulation of transmitter release in the CNS and the experimental systems used to study a functional receptor.

1.4 PRESYNAPTIC REGULATION OF TRANSMITTER RELEASE

1.4.1 Presynaptic receptors

In 1961, Koelle showed that in sympathetic ganglia ACh facilitated its own release by acting at receptors on the terminals from which it was released (Koelle, 1961). This demonstration provided the first evidence for functional presynaptic receptors. Since this early observation many different types of presynaptic receptors have been described (reviewed by Vizi, 1979; Langer, 1981; Starke, 1981; Chesselet, 1984).

Presynaptic receptors can be divided into two groups

i) Autoreceptors; receptors which respond to the neurotransmitter released from the nerve terminal; and,

ii) Heteroreceptors; receptors which are activated by neurotransmitters other than the native neurotransmitter released from the nerve terminal.

By definition, a presynaptic receptor is one which modulates neurotransmitter release and/or neurotransmitter synthesis (Lehmann and Langer, 1983). However, presynaptic receptors may not always be postsynaptic to another nerve cell e.g. in the striatum there is little evidence for axo-axonic synapses and it has been suggested that the interaction between ACh and DA terminals could occur by non-synaptic communication (see Bonanno *et al.*, 1985).

Presynaptic receptors have also been categorised according to whether they operate mainly under a) resting as well as depolarising conditions: Type I receptor-effector complexes (REC) or b) depolarising conditions: Type II REC (Lehmann and Langer, 1983).

This classification may also be used for receptors in addition to those on nerve terminals.

a) Type I REC

When these are activated a specific ion conductance is changed irrespective of the membrane's potential. They are present in muscle endplates, neuronal cell bodies and nerve terminals.

b) Type II REC

These typically act to regulate the conductance of ion channels which are opened in response to depolarisation (voltage-dependent ion-channels). *In vitro* depolarisation may be achieved by either field stimulation or by application of a high concentration of K^+. Type II REC are located on nerve terminals, cell bodies, smooth muscle and glands, and can modulate neurotransmitter release, rhythmic firing patterns in neurones and the force of muscle contraction.

Autoreceptors

The regulation of catecholamine release by presynaptic autoreceptors has received most attention (reviewed by Langer, 1981). The release of noradrenaline is modulated by inhibitory α_2-adrenoceptors and facilitatory β-adrenoceptors in a range of tissues in both the PNS and CNS. The release of DA in the CNS is subject to inhibition via presynaptic DA receptors.

In contrast, the presence of cholinergic autoreceptors is less well documented. In the PNS there is evidence for inhibitory muscarinic and facilitatory nicotinic autoreceptors in the

myenteric plexus (Briggs and Cooper, 1983). There is also evidence for inhibitory presynaptic muscarinic receptors in *Torpedo* electric organ (Dowdall *et al.*, 1982). Recently, facilitatory nicotinic autoreceptors (Rowell and Winkler, 1984; Moss and Wonnacott, 1985; Beani *et al.*, 1985) and inhibitory muscarinic autoreceptors (de Belleroche and Gardiner, 1982; Raiteri *et al.*, 1984; Meyer and Otero, 1985) have also been shown to exist in the CNS. Of particular interest is the reported nicotinic regulation of ACh release in the hippocampus (Moss and Wonnacott, 1985). This will be considered in greater detail in Chapter 5.

Hetereoreceptors

The modulation of neurotransmitter release by hetereo-receptors has also been extensively studied (reviewed by Starke, 1981; Langer, 1981; Chesselet, 1984).

The release of DA in the area of the brain known as the striatum has been shown to be regulated by a range of neurotransmitters. However, in this thesis attention will be mainly focused on the evidence for cholinergic modulation of DA release.

1.4.2 The nigro-striatal pathway

The nigro-striatal pathway is a neuronal route formed between the substantia nigra and the striatum (Fig. 1.5). The striatum is made up of the caudate nucleus and putamen, and together with the globus pallidus, subthalamic nucleus and substantia-nigra the complex known as the 'Basal ganglia' is formed (reviewed by Heimer *et al.*, 1985). Medically, the basal ganglia has been of great interest because of its involvement in neurological

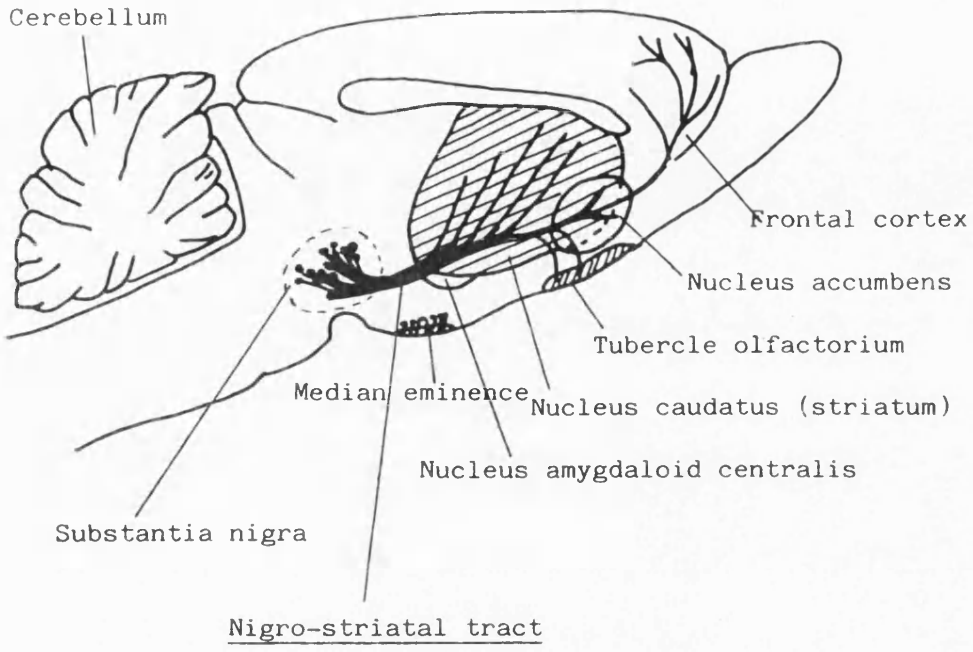


Fig. 1.5. Dopamine pathways in rat brain showing the Nigro-striatal tract.

disorders such as Parkinson's disease, Tardive dyskinesia and Huntington's chorea. The release of neurotransmitters and their regulation within the basal ganglia has therefore been an area of particular research.

The major neurotransmitter in the nigro-striatal pathway has been shown using histochemical and fluorescence techniques to be DA (Anden *et al.*, 1964). The striatum therefore contains a dense network of dopaminergic nerve terminals. The striatum also receives inputs from several other regions of the brain (see Graybiel and Ragsdale, 1983) and is therefore rich in many different neuro-transmitters. Another major transmitter in the striatum is ACh, which has been shown to originate from cholinergic interneurones (McGeer *et al.*, 1975) which are distributed throughout the striatum.

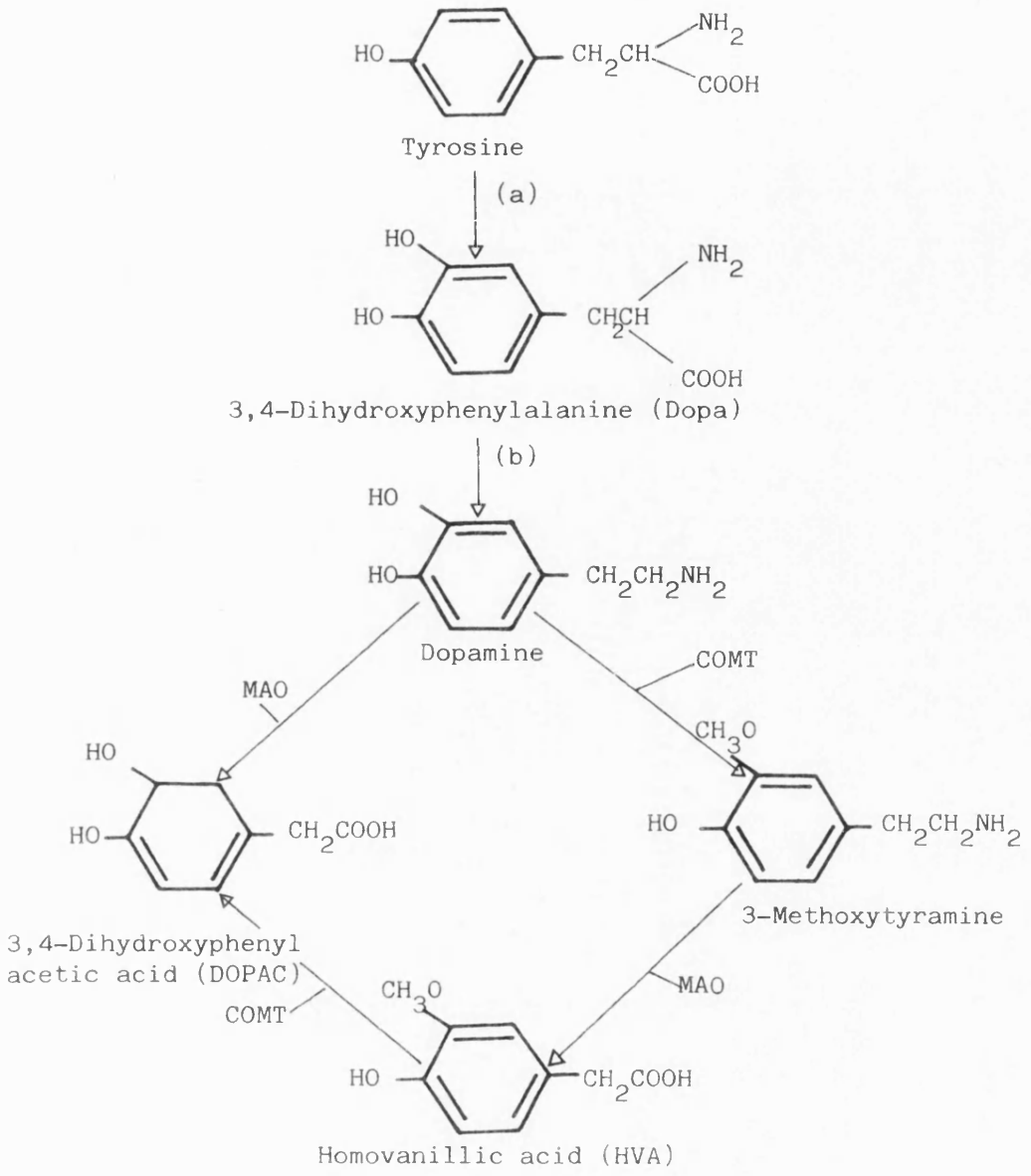
DA-ACh interactions in the striatum are well documented (Lehmann and Langer, 1983) and there is substantial evidence that DA plays a role in the regulation of ACh release and likewise ACh plays a significant role in the regulation of DA release.

1.4.3 Experimental procedures used to study the release of DA

The synthesis of DA and the regulation of its release from the nerve terminal are summarised in Figs. 1.6 and 1.7.

To study DA uptake and modulation of its release from the nerve terminal, striatal DA pools have been labelled directly using either [^{14}C] or [^{3}H]DA, taking advantage of the high affinity uptake process (Holz and Coyle, 1974). Alternatively, the precursor [^{3}H] tyrosine has been used, in which case [^{3}H]DA is synthesised within the terminal.

Fig. 1.6. Synthesis of dopamine and interrelationship of dopamine metabolites.



	Enzyme	cofactors
(a)	Tyrosine hydroxylase (E.C. 1.14.6.2)	Tetrahydrobiopterine, Molecular O_2, Fe^{2+}, NADPH
(b)	Aromatic L-amino acid decarboxylase (E.C. 4.1.1.28)	Pyridoxal phosphate
(c)	Monoamine oxidase (MAO) (E.C. 1.4.3.4)	FAD
(d)	Catechol-O-methyltransferase (COMT) (E.C. 2.1.1.6)	S-adenosylmethionine, divalent cations

Fig. 1.7. Regulation of the release of DA from the nerve terminal.

DA is synthesised from tyrosine (via L-Dopa) which is either stored in synaptic vesicles (1) or remains as a cytoplasmic pool. Upon arrival of the action potential, DA is either released from the cytoplasm (2), being metabolized by mitochondrial MAO to form DOPAC,or released from synaptic vesicles (3).

In the synaptic cleft DA either activates pre-synaptic autoreceptors (4), is taken up into the presynaptic terminal (5), metabolised by the combined action of MAO and COMT, to form 3-methoxytyramine (MT) and HVA (6) or interacts with the postsynaptic DA receptors (7)

MIT - mitochondrion

SV - synaptic vesicle

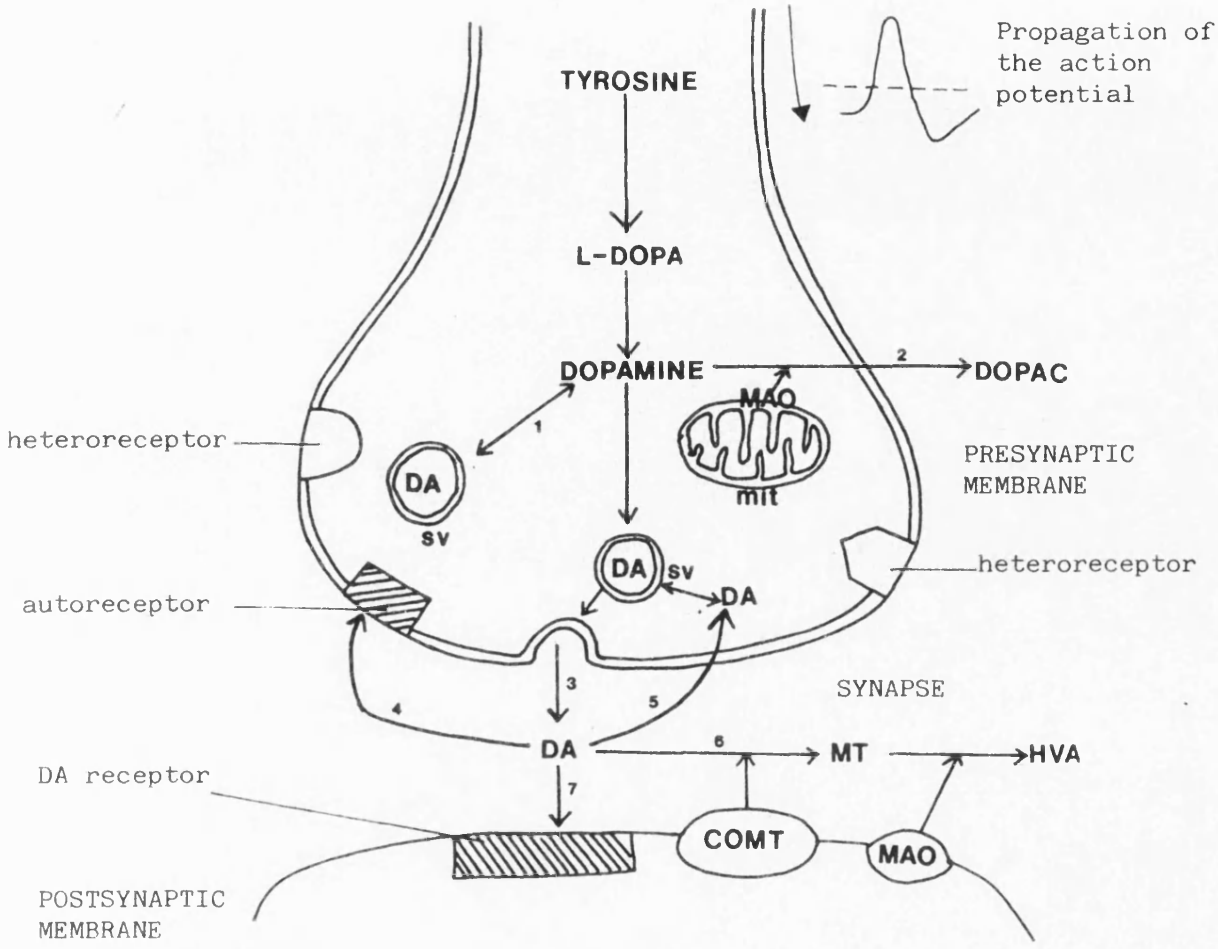


Fig. 1.7. Regulation of the release of DA from the nerve terminal.

a) Measurement of released transmitter

Two main types of experimental procedures have been used to monitor the efflux of radiolabelled transmitter in response to stimulation; i) static incubation, and ii) perfusion (superfusion). Static incubation has been employed by several groups (e.g. Futerman *et al.*, 1982; Connelly and Littleton, 1983) but the method has been criticised because it is not possible to completely prevent the reuptake by the tissue of the released transmitter (Raiteri *et al.*, 1974); this makes the interpretation of the release data difficult. In preference, the method of perfusion is usually now used. Perfusion involves the continuous washing of the tissue (either preloaded with radiolabel, e.g. de Belleroche and Bradford, 1978; Lehmann and Langer, 1982, or the continuous perfusion with radiolabel, e.g. Giorguieff *et al.*, 1976, 1977, 1979) and the collection of serial fractions of the effluent. Administration of stimulating and blocking agents into the main flow is relatively easy and reuptake of the released transmitter is less likely than in the static incubation system. Perfusion studies have been carried out *in situ* (e.g. Giorguieff *et al.*, 1976, where perfusion of cat striata was carried out through a push-pull cannula) or *in vitro* using a) slice preparation (e.g. Giorguieff *et al.*, 1976; Lehmann and Langer, 1982) or b) a synaptosomal preparation (e.g. de Belleroche and Bradford, 1978; Mills and Wonnacott, 1984).

b) Slice preparations

Many studies using brain slice preparations have been carried out after the work of McIlwain, (1961) who developed

a technique of slicing the brain to produce preparations which were viable for several hours. Brain slices can be prepared either by hand or mechanically and ideally should not be thicker than 0.35 mm. They are highly organised structures containing both neuronal and non-neuronal cells. They have several advantages, including their ability to be electrically stimulated and be used in electrophysiological studies. The application of brain slices to cholinergic biology has been reviewed by Weiler *et al.* (1982). Their use in the study of the cholinergic modulation of DA release in the striatum is summarised in Table 1.2.

c) Synaptosomal preparations

When brain tissue is homogenised, presynaptic nerve terminals are detached and form sealed structures known as "synaptosomes". These subcellular particles were originally isolated by density gradient centrifugation techniques (Gray and Whittaker, 1962; De Robertis *et al.*, 1962).

Synaptosomes have several morphological characteristics; they appear in section as membrane-bounded oval profiles about 0.5 - 2 μm in diameter containing numerous vesicles, small mitochondria and in many cases an attached postsynaptic membrane (reviewed by Jones, 1975; Whittaker, 1984). Synaptosomes are of particular value because they survive the homogenisation and centrifugation procedures and remain in iso-osmotic medium as active metabolic units for several hours (Bradford *et al.*, 1975). Normal ionic gradients are maintained across the membrane and the synaptosomes display the ability to synthesise and store transmitter substances. A synaptosomal preparation is therefore particularly useful in the

study of presynaptic neurochemical events.

There are several advantages and disadvantages in using either a slice or synaptosomal preparation for the study of the presynaptic regulation of transmitter release. These will be discussed later (p.181).

1.4.4 Presynaptic modulation of DA release in the striatum

a) Autoreceptors

The activation of DA autoreceptors results in an inhibition of the released DA. These autoreceptors typically act under depolarising (either electrical or K^+-evoked) conditions (e.g. Arbilla and Langer, 1984) and are therefore Type II REC (after Lehmann and Langer, 1983). The mechanism by which they act has recently been shown to involve the inhibition of tyrosine hydroxylase activity (El Mestikawy *et al.*, 1986).

b) Cholinergic heteroreceptors

Early evidence for the regulation of DA release from the striatum by ACh was provided by Besson *et al.* (1969) who demonstrated that at a low concentration ACh (10 µM) stimulated the release of [^{3}H]DA synthesised from [^{3}H]tyrosine in the isolated striatum. Since then there have been many similar demonstrations (see Table 1.2). However, the type of response (either facilitatory or inhibitory) caused by either nicotinic or muscarinic agents was controversial. It was generally found that nicotinic agents enhanced DA release in either stimulated or unstimulated tissue (e.g. Sakurai *et al.*, 1982). Facilitatory nicotinic receptors are therefore clearly present on the dopaminergic nerve terminals and

Table 1.2. Summary of the evidence for the cholinergic modulation of DA release in the striatum using perfusion techniques

Enhancement by agonist of either basal or stimulated release (↑), reduction (↓) and no effect (x).

Level of antagonism, complete (✓), partial (~) or ineffective (X)

Preparation	Effect on Basal or stimulated release	Agonist	Response	Antagonist	Level of Antagonism	Group
NICOTINIC						
SLICE	Basal	Nicotine (5 mM)	↑	Hexamethonium (9.3 μM)	~	Westfall (1974a)
"	Basal	Nicotine (100-1000 μM) DMPP (50-750 μM)	↑	 Hexamethonium (10 μM)	ND ~	Westfall *et al.* (1983a)
"	Basal	ACh (10 μM)	↑	Mecamylamine (10 μM)	~	Giorguieff *et al.* (1976)
"	Basal	ACh (10 μM)	↑	Pempidine (10 μM)	~	Giorguieff *et al.* (1977)
"	Basal	Nicotine (1 μM)	↑	Pempidine (10 μM) d-tubocurarine (5 μM)	✓ ~	Giorguieff-Chesselet *et al.* (1979)
"	Basal	Nicotine (1 mM)	↑		ND	Arqueros *et al.* (1978)
"	Basal Basal	Nicotine (3 mM) Nicotine (5 μM)	↑ ↑	Hexamethonium (0.3 mM)	X ND	Marien *et al.* (1983) Arbuthnott *et al.* (1984)
SYNAPTOSOMAL	K^+ (56 mM)	ACh (0.3 mM)	↑	Hexamethonium (0.2 mM) α-BGT (0.188 μM)	~ ~	de Belleroche and Bradford (1978)
"	Basal	Nicotine (200-500 μM) ACh (500 μM) Lobeline (10-100 μM) Coniine (50-200 μM)	↑ ↑ ↑ ↑	 d-tubocurarine (1 mM) hexamethonium (0.1 mM) 	ND ~ X ND	Sakurai *et al.* (1982)
"	K^+ (25 mM)	Lobeline (50 μM)	↑		ND	Sakurai *et al.* (1982)
"	Basal	Nicotine (100 μM)	↑	Mecamylamine (100 μM)	✓	Mills and Wonnacott (1984)
PUSH-PULL CANNULA	Basal	ACh (10 μM)	↑	Hexamethonium (10 μM) Mecamylamine (10 μM)	~ ~	Giorguieff *et al.* (1976)

Table 1.2 (continued)

Preparation	Effect on Basal or stimulated release	Agonist	Response	Antagonist	Level of Antagonism	Group
MUSCARINIC						
SLICE	Nicotine (5 mM)	ACh (10 μM)	↑		ND	Westfall (1974a)
"	K^+ (50 mM)	ACh (1-10 μM)	↑		ND	Westfall (1974b)
"	Electrical	ACh (1-100 μM)	↑		ND	Westfall (1974b)
"	Basal	ACh (10 μM)	↑	Atropine (1 μM) Scopolamine (1 μM)	~ ~	Giorguieff *et al.* (1977)
"	Basal	Oxotremorine (10μM)	↑	Atropine (1 μM)	✓	Giorguieff *et al.* (1977)
"	Electrical	Oxotremorine (1.8-10 μM)	↑	Atropine (0.1 μM)	✓	Lehmann and Langer (1982)
SYNAPTOSOMAL	K^+ (56 mM)	ACh (0.3 mM)	↑	Atropine (0.129 mM)	~	de Belleroche and Bradford (1978)
"	Basal	Oxotremorine (20 μM)	X			Sakurai *et al.* (1982)
"	K^+	Oxotremorine (50 μM)	X			Sakurai *et al.* (1982)
"	K^+ (15 mM)	ACh (10 μM)	↑	Atropine (0.1 μM)	✓	Raiteri *et al.* (1982)

can be classified as 'Type I REC' (after Lehmann and Langer, 1983). ACh evoked transmitter release was partially blocked by mecamylamine, pempidine, hexamethonium, α-BGT and d-tubocurarine (Giorguieff *et al.*, 1976; de Belleroche and Bradford, 1978; Sakurai *et al.*, 1982) whereas nicotine evoked release was completely blocked by pempidine, and mecamylamine (Giorguieff-Chesselet *et al.*, 1979; Mills and Wonnacott, 1984) and partially blocked by hexamethonium (Westfall, 1974a).

The inability of nicotinic antagonists to completely block the release evoked by ACh suggested the possibility of facilitatory muscarinic receptors on dopaminergic nerve terminals. The reported action of muscarinic agonists on the release of DA is mixed (Westfall, 1974a,b; de Belleroche and Bradford, 1978; Giorguieff *et al.*, 1976) and the differences are dependent upon the experimental conditions employed. Westfall (1974a,b), and de Belleroche and Bradford (1978), looked at the effect of ACh on stimulated release of transmitter from striatal slices and both reported that muscarinic receptors were inhibitory. In contrast, Giorguieff *et al.* (1977) reported that ACh evoked the release of [^{3}H]DA from unstimulated slices and that this was partially blocked by the muscarinic antagonists atropine and scopolamine, suggesting that muscarinic receptors were facilitatory. More recent studies have shown that activation of muscarinic heteroreceptors by ACh potentiates stimulated (electrical or K^+ evoked) release of [^{3}H]DA (Lehmann and Langer, 1982; Raiteri *et al.*, 1982). Muscarinic hetero-receptors therefore fall into the category of 'Type II REC'. The facilitatory effect of ACh on [^{3}H]DA release was also shown to be dependent upon the concentration of K^+ used to depolarise the nerve

endings. Raiteri *et al.* (1984) reported an optimum concentration of 15 mM should be used. This may explain the discrepancies in the literature concerning the influence of ACh on DA release (mentioned above), where high concentrations of K^+ (56 mM) were used by de Belleroche and Bradford (1978) and a high depolarising nicotine concentration (5 mM) by Westfall (1974a). Besides enhancing stimulated [^{3}H]DA release, muscarinic agents have also been shown to increase the basal (spontaneous) release of newly synthesised [^{3}H]DA (Giorguieff *et al.*, 1977). This contradicts the classification of these receptors as type II REC and emphasises the limitations of this general classification. That muscarinic heter oreceptors may be stimulated by endogenous levels of ACh was shown by the reduction of evoked release of DA when muscarinic antagonists in addition to anticholinesterases were present (Lehmann and Langer, 1982). In this type of experiment, electrical stimulation evokes the release of transmitter from all nerve terminals and therefore it is not surprising that the levels of ACh reach a level which will activate muscarinic receptors. However, under similar conditions activation of nicotinic receptors was inferred not to occur by the lack of effect of d-tubocurarine on evoked release (Lehmann and Langer, 1982), although antagonism of nicotinic responses in the CNS by d-tubocurarine has not been convincingly demonstrated (Krnjević, 1975).

There are therefore several problems in studying muscarinic and nicotinic events separately, using ACh as an agonist, and a heterogeneous population of nerve terminals. For example, when atropine is used as a muscarinic antagonist both muscarinic heteroreceptors on dopaminergic nerve terminals and inhibitory

muscarinic autoreceptors on cholinergic nerve terminals will be blocked. This will lead to an increase in the concentration of ACh which will activate nicotinic receptors on dopaminergic nerve terminals leading to an increase in DA release. To completely block the action of ACh it is therefore necessary to have both nicotinic and muscarinic antagonists present (Giorguieff *et al.*, 1977).

Overall, the results from both types of release study *in vitro* (synaptosomal and slice) have been similar (see Table 1.2). From the experiments *in situ* there is also evidence for a nicotinic facilitatory mechanism (Giorguieff *et al.*, 1976) although a muscarinic involvement has yet to be demonstrated. There is however indirect evidence for a muscarinic effect as muscarinic agents have been shown to increase DA turnover in the striatum (Westerlink and Korff, 1976).

Although the use of a synaptosomal preparation strongly suggests that the regulatory cholinergic receptors are present on the dopaminergic nerve terminals, in a slice preparation where there are many neuronal interconnections, the exact location of the receptors is less clear. Perfusion in the presence of TTX, a specific inhibitor of the voltage sensitive sodium channel (discussed later in Section 3.3.4c) showed that the action of cholinergic agents was independent of the activity of the nerve (Giorguieff *et al.*, 1977) and suggested that the cholinergic receptors modulating DA release in a slice preparation were situated at the nerve terminal.

Further evidence for a presynaptic location of nicotinic receptors on dopaminergic nerve terminals was shown by measurement of a reduction in [^{125}I]α-BGT binding sites in the striatum

following 6-hydroxydopamine-induced lesions of the nigro-striatal pathway (de Belleroche *et al.*, 1979). More recent studies using [^{3}H] ACh (Schwartz *et al.*, 1984) and [^{3}H]nicotine (Clarke and Pert, 1985) as binding ligands have confirmed this finding. Although a decrease in muscarinic sites has also been shown using a similar approach, the evidence for a presynaptic location is less convincing (Chesselet, 1984).

To summarise, it has been shown that both muscarinic and nicotinic cholinergic receptors are present on dopaminergic nerve terminals in the striatum and they play a facilitatory role in the regulation of DA release. The nicotinic regulation of DA release therefore provides a system to study functional nAChR. However, the pharmacology of this regulation is not clear; both C_6 and C_{10} nicotinic antagonists have been reported to inhibit the action of nicotinic heteroreceptors (see Table 1.2).

The aim of this project was to develop a perfusion system which could be used to study the nicotinic regulation of striatal DA release in more detail. A synaptosomal preparation was chosen instead of a slice preparation so that the regulation of transmitter release at the nerve terminal could be studied without the possible involvement of striatal interneurones. To monitor the release of DA the striatal synaptosomes were preloaded with [^{3}H]DA. [^{3}H]Tyrosine was not used because this would require isolation of the DA from the perfusate.

The following chapter describes the characterisation of the striatal synaptosomal preparation and the optimisation of the DA uptake conditions. The subsequent chapter then summarises the development of a sensitive perfusion system in which synaptosomes

may be repeatedly stimulated using micromolar concentrations of nicotinic agonists. The revised system was then used to study the effect of nicotinic antagonists on agonist and K^+-evoked [^{3}H]DA release. Finally, for comparison the nicotinic autoreceptor present on cholinergic hippocampal nerve terminals was studied by the perfusion of hippocampal synaptosomes preloaded with [^{3}H]choline.

MATERIALS

Chemicals

Unless otherwise stated below, all general reagents were supplied by either BDH (Poole, Dorset, U.K.), Fisons (Loughborough, Leicestershire, U.K.) or Sigma Chemical Company Ltd. (Poole, Dorset, U.K.).

Lobeline hydrochloride, 1-1-dimethyl-4-phenylpiperazinium and diphenylborate-ethanolamine were from Aldrich Chemical Co. Ltd. (Gillingham, Dorset, U.K.).

Cytisine was from Koch-Light Laboratories Ltd. (Colnbrook, Berks, U.K.).

Tetraoctylammonium bromide was obtained from Fluorochem Ltd. (Glossop, Derbyshire, U.K.).

(-)-Nicotine hydrogen (+)-tartrate was from BDH (Poole, Dorset, U.K.).

α-Bungarotoxin was obtained from Boehringer Corporation (Lewes, Sussex, U.K.).

Acetylcholine chloride, carbamylcholine chloride, choline chloride, decamethonium bromide, 3,4-dihydroxyphenylethylamine hydrochloride (dopamine), homovanillic acid, hexamethonium bromide, lactate dehydrogenase (E.C. 1.1.1.27), L-nicotine, oxotremorine, pargyline, tetrodotoxin, D-tubocurarine chloride and veratridine were from Sigma Chemical Company Ltd. (Poole, Dorset, U.K.).

Drugs which were gifts

Chlorisondamine from Dr. I. Stolerman, Institute of Psychiatry, (London, U.K.).

Dihydro-β-erythroidine hydrobromide from Dr. Benifeld, Merck, Sharp

and Dohme Research Lab. (Hertfordshire, U.K.).

Ketamine from Parke, Davis and Co. (Cambridge, U.K.).

Mecamylamine hydrochloride from Dr. Caulfield, Glaxo Group Research Ltd. (Greenford, U.K.).

Neosurugatoxin from Professor T. Kosuge, Shizuoka College of Pharmacological Sciences, (Shizuokay, Japan).

(+)-Nicotine di (-)-tartrate from Dr. R. Barlow, University of Bristol (Bristol, U.K.).

Nomifensine maleate from Dr. Schone, Hoechst (Frankfurt, Germany).

Pempidine tartrate from Mrs. Adams, May and Baker Ltd. (Dagenham, Essex, U.K.).

Perhydrohistrionicotoxin from Dr. E.X.Albuquerque, University of Maryland (Baltimore, U.S.A.).

Stock Solutions

Dihydro-β-erythroidine hydrobromide (25 mg) was dissolved in 1.82 ml ethanol (95%) to give a final concentration of 50 mM. This solution was stored at 4°C.

Veratridine was dissolved in 50% ethanol to give a final concentration of 20 mM and stored at 4°C.

Ketamine was dissolved in water to give a concentration of 10 mM and stored at 4°C (Maleque *et al.*, 1981).

Tetrodotoxin (TTX, 1 mg + 5 mg sodium acetate) was carefully dissolved in water to form a 1 mM solution. Aliquots (100 µl) were stored at -80°C until required.

α-Bungarotoxin (α-BGT, 1 mg) was dissolved in the appropriate buffer to a concentration of 1 µM and stored at -20°C in 100 µl aliquots.

Pure crystalline neosurugatoxin (250 µg) was dissolved in 0.12 ml dimethylsulphoxide and diluted with 2.88 ml water to give a 0.1 mM solution (Kosuge *et al.*, 1982). Aliquots (50 µl) were stored at -80°C until required.

Pérhydrohistrionicotoxin dissolved in 0.1 ml ethanol to 10 mM was stored in 20 µl aliquots at -80°C.

Storage and dilution of Pharmacological agents for perfusion experiments

All drugs were stored at 4°C (unless otherwise stated). Each drug was dissolved in perfusion medium (p.135) and the pH adjusted to 7.4 if necessary. Concentration ranges of drugs were obtained by serial dilution of the highest concentration.

Radiolabelled Compounds

[^{3}H]Dopamine

[7,8-^{3}H] Dopamine (46 Ci/mmol) was obtained from Amersham International (Amersham, Bucks, U.K.). It was diluted 1:1 with 2.5 mM ascorbic acid and stored at -20°C in 50 µl aliquots.

[^{3}H]Choline

Methyl-[^{3}H]choline chloride (78 Ci/mmol) was supplied by Amersham International (Amersham, Bucks, U.K.). It was diluted 1:1 with 20 µM choline chloride and stored at -20°C in 100 µl aliquots.

[^{3}H]Nicotine

(+/-) N-methyl-[^{3}H]nicotine (73.7 Ci/mmol) was purchased from NEN Radiochemicals Ltd. (Southampton, Hants, U.K.) and stored in

ethanol at -20°C.

[^{125}I] α-Bungarotoxin

α-Bungarotoxin (α-BGT) was iodinated in the laboratory following the method of Wonnacott *et al.*, (1980), using carrier-free Na [^{125}I] obtained from Amersham International (Amersham, Bucks,U.K.). Using this method about 80% of the iodinated toxin was the mono derivative. [^{125}I]α-BGT was stored at 4°C for not longer than 3 weeks.

Other Materials

Glass fibre filters were from Whatman Biochemicals Ltd. (Maidstone, Kent, U.K.). Aqua Luma was from LUMAC B.V., (Ad Schaesberg, The Netherlands). Optiphase Safe was from LKB Instruments (South Croydon, Surrey, U.K.). Picofluor TM30 and Emulsifier 299TM were from Packard Instruments Ltd. (Pangbourne, Berks, U.K.). Perfusion apparatus (see p.**136**).

CHAPTER 2

PREPARATION AND CHARACTERISATION OF RAT BRAIN STRIATAL SYNAPTOSOMES

2.1 INTRODUCTION

Traditional methods for preparing synaptosomes take almost 3 h (Gray and Whittaker, 1962) although many modified procedures have since been described (reviewed by Jones, 1975; Gibson and Blass, 1982). The method used in the work described in this thesis was based on that of de Belleroche and Bradford (1980) which takes approximately 1½ h. However, the procedure was modified (as reported in Section 2.2.1) so that the process consisted of just one high speed centrifugation step (1 h) followed by a low speed wash (7 min). The purity of the synaptosomal fraction was assessed by electron microscopy using both negative and positive staining techniques. To determine the integrity of the synaptosomal preparation the cytoplasmic enzyme marker lactate dehydrogenase (E.C. 1.1.1.27) was assayed and the high affinity uptake of [^{3}H]DA was measured,confirming the presence of dopaminergic nerve endings. The conditions for loading the striatal synaptosomes with [^{3}H]DA were then studied to ensure optimum usage of tissue, radiolabel and time.

The subcellular distribution of [^{3}H]nicotine and [^{125}I]α-BGT binding sites was also determined to demonstrate that nicotinic binding sites are enriched in striatal nerve terminal preparations.

2.2 METHODS

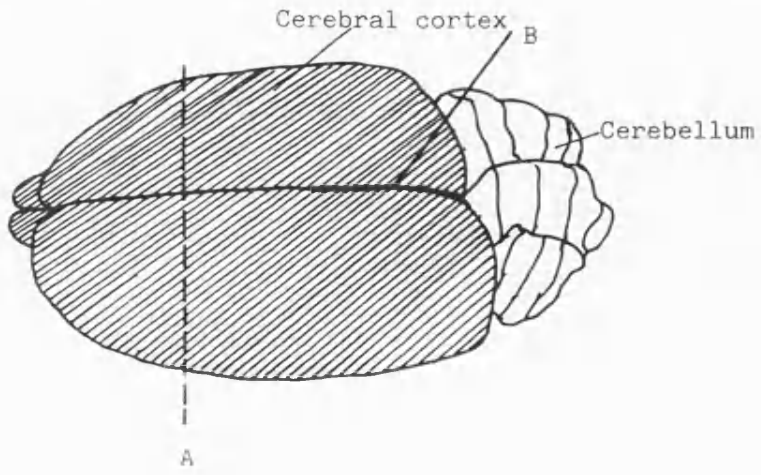
2.2.1 Preparation of synaptosomes

Male Wistar rats weighing 150-200g were killed by decapitation. The brains were rapidly removed and placed on filter paper moistened with ice cold 0.32 M sucrose, pH 7.4. The striata were dissected out following the method of Glowinski and Iversen (1966; Fig 2.1). Striata from 2 rats (70-90 mg wet wt) were homogenised in 0.32 M sucrose, pH 7.4, 10% (w/v), in a precooled glass-teflon homogeniser (clearance 0.31 mm) by 12 up and down strokes at 200 rpm in a time of 30 s. The homogenate was diluted with 0.32 M sucrose to approximately 4 ml and layered onto two discontinuous sucrose gradients prepared from 1.2 M sucrose (2 ml) overlaid with 0.8 M sucrose (2 ml) The gradients were centrifuged in a Beckman L5-50B ultracentrifuge equipped with a SW50.1 rotor, at 100,000 *g* for 1 h at 4°C. After centrifugation the band at the 0.8 M/1.2 M sucrose interface (Fig 2.3, p. **78**) was removed using a Pasteur pipette and slowly diluted (to avoid lysis of the synaptosomes) with an equal volume of perfusion medium (p.**135**). The diluted synaptosomal preparation was centrifuged in an MSE bench centrifuge at 1,000 *g* for 7 min at room temperature and the resulting pellet resuspended in perfusion medium, (1 ml medium/100 mg wet wt. original tissue).

2.2.2. Preparation of subcellular fractions for electron microscopy

a) Positive staining

Striata from 3 rat brains were homogenised and fractionated as described above. The 2 gradient bands (P_a and P_b see Fig. 2.3) and the pellet (P_c) were gradually diluted with 0.32 M sucrose followed by ultracentrifugation at 100,000 *g* for 1 h. The resulting pellets were



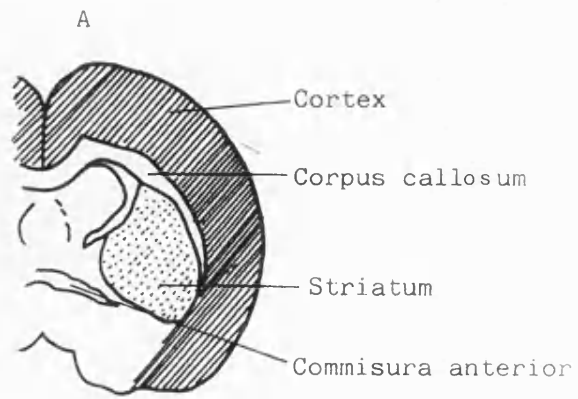
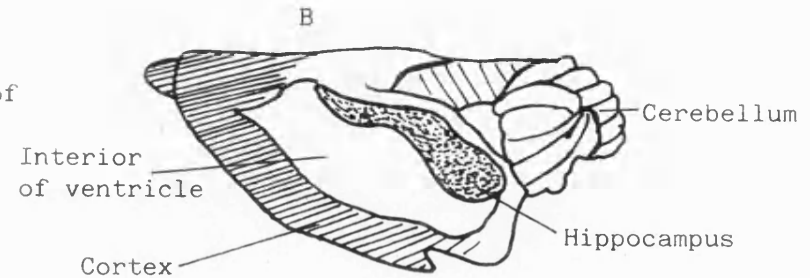
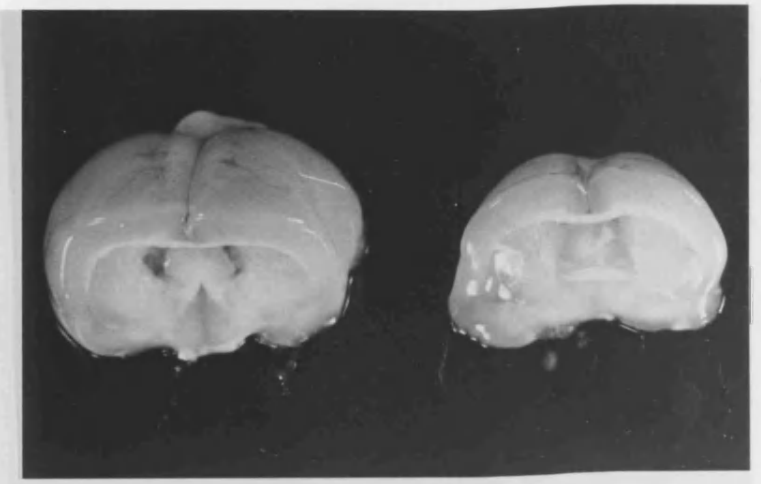


Fig. 2.1. Diagrammatic representation of the dissection procedure for rat brain following the method of Glowinski and Iversen (1966). Dotted line (A) indicates the position of section to reveal the striata. The hippocampus was dissected by pulling away the cerebral cortex from the mid line (B).

Opposite are photographs showing the dissection steps for the striata (ii) and the hippocampus (iii).



i. Whole rat brain



ii. Sectioned rat brain showing striata

iii. Side view of rat brain

iv. Dissected brain showing hippocampus on one side

fixed in 4% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at 4°C overnight. Samples (approximately 3 mm^3) were washed twice in the cacodylate buffer and then post fixed in 1% (v/v) osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2) for 1 h at room temperature. The samples were washed with distilled water followed by the addition of either 2% aqueous uranyl acetate or 1% tannic acid, buffered in 0.1 M cacodylate, for 1h in the dark at 4°C. After a further wash with distilled water, the specimens were dehydrated by passage through a graded series of acetone: water mixtures (30% - 100% acetone). The dehydrated pellets were transferred to 50% (v/v) Taab embedding resin in acetone and left overnight at room temperature. The samples were impregnated with 100% resin (two changes) over 48h and embedded at 60°C for the following 48h. Ultrathin sections were cut using an LKB ultramicrotome equipped with a glass knife and the sections were collected on copper grids. Finally, the specimens were stained with uranyl acetate followed by Reynolds lead citrate (2% aqueous) and examined with a Jeol 100 CX electron microscope at 80 kV.

b) Negative staining

The band isolated from the sucrose gradient at the 0.8/1.2 M sucrose interface (Fig. 2.3) was removed and gradually diluted with 2 volumes of 0.32 M sucrose (pH 7.4) followed by centrifugation at 1,000 *g* for 7 min at room temperature. The resulting pellet was resuspended in 2 volumes of 0.32 M sucrose (pH 7.4).

A drop of the suspension was placed on a copper, pioloform-coated grid and the grid then removed from the suspension. Excess fluid was drawn off the grid with a filter paper, and the grid dried under a lamp for 10 min. A drop of 25% (w/v) ammonium molybdate solution was

placed on the grid and the grid removed and dried as before. The sample was then visualised under the electron microscope as in positive staining.

2.2.3 Measurement of Lactate dehydrogenase (E.C.1.1.1.27) activity

Lactate dehydrogenase (LDH) can be used as a cytoplasmic marker (Johnson and Whittaker, 1963) and is frequently used to measure the integrity of a synaptosome preparation by initially measuring the free LDH activity and then the total activity after rupture of the synaptosomes with Triton X-100. Enzyme activity was assayed by measuring the oxidation of $NADH_2$ in the presence of pyruvate:

$$\text{Pyruvate} + \text{NADH} + H^+ \underset{\longleftarrow}{\overset{\text{LDH}}{\longrightarrow}} \text{Lactate} + NAD^+$$

By monitoring the rate of change of absorbance at 340 nm the LDH activity can be calculated as follows:

μmol pyruvate reduced/min/mg protein

$$= \frac{\Delta A}{E \times 1} \times \frac{\text{vol. in cuvette}}{\text{vol. in sample}} \times \frac{1}{\text{protein concentration}}$$

where ΔA = change in absorbance/min

E = molar absorption coefficient (6.22×10^3)

1 = length of light path (1 cm)

The occluded LDH activity was calculated as the difference between the total and free LDH activities.

The method used was that of Johnson (1960) with the modification by Marchbanks (1967). All reagents used for the assay were freshly prepared. In a 3 ml cuvette was placed 0.15 M Tris-HCl buffer pH 7.4,

(2.6 ml),2 mM NADH (0.1 ml) and a sample (25 µl) from the sucrose density gradient. The cuvette was placed in a Pye Unicam SP6-450 spectrophotometer equipped with a chart recorder and the reaction was initiated by the addition of 10 mM pyruvate (0.1 ml) followed by rapid mixing. Free LDH activity was measured for at least 1 min after which the total activity was measured by the addition of 0.1 ml 10% (v/v) Triton X-100 to the assay mixture. All assays were carried out in duplicate and the results averaged.

2.2.4 Determination of Protein

Protein was assayed by the method of Lowry *et al.* (1951). Standard curves were constructed using a freshly prepared solution of bovine serum albumin (BSA) over the concentration range 0-250 µg/ml. BSA standards (200 µl) or unknown (200 µl synaptosome preparation diluted 1/40 with distilled water) were incubated with 1.0 ml alkaline cupric tartrate, freshly prepared by diluting 1 vol. of 1% (w/v) sodium, potassium tartrate and 1 vol. of 0.5% (w/v) $CuSO_4.5H_2O$ with 50 vols. of 2% (w/v) Na_2CO_3 in 0.1M NaOH. After incubation for 10 min at room temperature, Folin-Ciocalteau's reagent (0.1 ml) diluted 1:1 with water was added to each sample and the colour allowed to develop for 30 min. Blanks contained distilled water in place of protein. The absorbance was read at 750 nm in a Pye Unicam SP 8-100 spectrophotometer.

2.2.5 Binding of [^{125}I]αBGT and [^{3}H]nicotine to rat brain membranes

a) Preparation of rat brain P_2 membranes

Brains were dissected, minus cerebellum, from male Wistar rats (200 g) and stored at -80°C. Frozen tissue, approximately 6 g, was

allowed to thaw in 0.32 M sucrose, pH 7.4, containing 1 mM EDTA, 0.01% (w/v) sodium azide and 0.1 mM phenylmethylsulphonylfluoride (PMSF). A 10% (w/v) homogenate in 0.32 M sucrose was prepared using a Teflon homogeniser (Aldridge *et al.*, 1960), 6 strokes at 800 rpm in 15 s. The homogenate was centrifuged at 1,000 *g* for 10 min at 4°C. The supernatant was removed and placed on ice. The pellets were resuspended in 0.32 M sucrose to approximately half the original volume and recentrifuged at 1,000 *g* for 10 min at 4°C. The combined supernatants were centrifuged at 20,000 *g* for 30 min at 4°C. Using a Pasteur pipette the final pellet (P_2) was resuspended in 50 mM - phosphate buffer, pH 7.4, containing 1 mM EDTA, 0.1 mM PMSF and 0.01% (w/v) sodium azide, 2 ml/g original wet wt. The final suspension was filtered through muslin and the filtrate stored at 4°C. The approximate protein concentration was 10 mg/ml as determined using the method of Lowry *et al.* (1951), (p. **68**).

b) Preparation of striatal synaptosomal membranes

Synaptosomal membranes were prepared from striata which had been stored at -80°C. Tissue, from 6 rats, was allowed to thaw in 0.32 M sucrose, pH 7.4, at 4°C for 20 min. A 10% (w/v) homogenate was prepared and subjected to isopycnic density gradient centrifugation as described in Section 2.2.1 (p. **63**). The tissue at the 0.8/1.2 M interface was removed and diluted with an equal volume of 50 mM sodium phosphate buffer, pH 7.4, (see "Preparation of rat brain P_2 membranes"). The diluted membranes were centrifuged at 1,000 *g* for 7 min at room temperature and the resulting pellet resuspended in the phosphate buffer allowing 1 ml/100 mg wet wt. original tissue. The final protein concentration was 2-2.5 mg/ml.

c) Measurement of [^{125}I]α-BGT binding sites

[^{125}I]α- BGT binding to rat brain membranes was assayed by the method of Schmidt (1977). Membrane preparations were diluted with 50 mM phosphate, pH 7.4, containing 1 mM EDTA, 0.01% (w/v) sodium azide and 0.1% (w/v) PMSF, to give a protein concentration of 0.5 - 2 mg/ml. Samples (500 µl) of the diluted tissue were incubated with [^{125}I]α-BGT (see "Materials"), 5 nM, unless otherwise stated in Eppendorf tubes for 60 min at 22°C. Non-specific binding was determined by incubation of the membranes in the presence and absence of 0.5 µM α-BGT and the specific binding was defined as the difference between the total and non specific binding. Bound radioligand was separated by dilution of the samples with 1 ml phosphate-buffered saline (PBS), pH 7.4 containing 10 mM potassium phosphate, 150 mM sodium chloride and 0.01% (w/v) sodium azide. The diluted samples were centrifuged in a MSE Microcentaur bench centrifuge at 10,000 *g* for 2 min. The supernatant was removed using a Pasteur pipette and the pellet washed by resuspension in 1.5 ml ice cold PBS, followed by centrifugation at 10,000 g for 2 min. Pellets were counted for radioactivity (p. **74**). All assays were carried out in triplicate and blanks contained 50 mM phosphate, pH 7.4, in place of sample. The binding of [^{125}I]α-BGT was measured over the concentration range 0.2 - 10 nM.

d) Measurement of [^{3}H]nicotine binding sites

The binding of [^{3}H]nicotine to rat brain membranes was carried out as described by Romano and Goldstein (1980). The membrane preparations were diluted with 20 mM HEPES buffer, pH 7.5, containing 118 mM NaCl, 48 mM KCl, 2.5 mM $CaCl.2H_2O$ and 1.2 mM $MgSO_4.7H_2O$. The P_2 membranes were diluted to 1.3 - 2.6 mg protein/ml. The striatal

synaptosomal membranes were diluted to 0.5 - 0.6 mg protein/ml because of the low amount of tissue available.

Samples (250 µl) of the diluted membranes were incubated with 40 nM [^{3}H](+/-)-nicotine for 60 min at 22°C in the presence and absence of 100 µM (-)-nicotine. After the incubation period 2 ml of ice cold 50 mM HEPES buffer, pH 7.5, were added to the membranes. Bound radioligand was immediately separated by filtration under vacuum through Whatman GFC filters (2.5 cm), presoaked in 0.1% (w/v) poly-L-lysine for 3h to reduce the non-specific binding of nicotine to filters (Romano and Goldstein, 1980). The sample tubes and filters were washed with 3 ml HEPES buffer at 4°C. The radioactivity remaining on the filters was counted (p.**74**). All assays were carried out in triplicate and blanks contained assay buffer in place of sample. [^{3}H] Nicotine binding to P_2 membranes was measured over the concentration range 3-100 nM and to synaptosomal membranes over the range 1-120 nM.

2.2.6 Analysis of the uptake of DA by striatal synaptosomes

Experiments were carried out to study DA uptake by striatal synaptosomes following the methods described by Schoemaker and Nickolson, (1983). Radiolabelled DA of high specific activity (46 Ci/mmol, [7, 8-^{3}H]DA, diluted as described in the "Materials" to give 10^5 dpm/pmole) was used to label the synaptosomal transmitter pools.

a) Uptake kinetics

Samples (1 ml) of synaptosomes diluted with perfusion medium to give a protein concentration of approximately 1 mg/ml were preincubated in Eppendorf tubes at 37°C for 10 min before [^{3}H]DA (diluted with unlabelled DA) was added. The uptake of DA was studied

over the concentration range 0.01 - 10 μM. The initial uptake was taken as that which occurred in the first minute of incubation at 37°C; for each DA concentration the initial uptake was measured in duplicate and the results averaged.

A 50 μl sample was removed and counted to give the total radioactivity present in the suspension. The remaining synaptosomes were separated from the medium by filtration under vacuum through glass fibre filters (Whatman GF/F) using a Millipore manifold unit. Each filter was washed with 10 ml ice cold medium, washing being completed in approximately 3 s. Non-specific uptake was determined by including the DA uptake inhibitor nomifensine (50 μM) in the medium. Non-specific uptake was also measured at 4°C and time zero with filtration and washing immediately following the addition of DA. The sodium dependency of the DA uptake was tested by incubation in sodium depleted medium (p.**137**). The radioactivity in each sample was counted (p. **73**) and the uptake calculated. Values were corrected for non-specific uptake and expressed as pmoles DA.mg protein $^{-1}$. min $^{-1}$.

b) Time course for the uptake of DA

Synaptosomes resuspended in perfusion medium (p.**135**) were preincubated at 37°C for 10 min prior to the addition of [^{3}H] DA, final concentration 0.11 μM. The uptake was followed by removing samples (100 μl) from the incubating suspension at time intervals after the addition of the radiolabel. The synaptosomes were rapidly separated from the medium by vacuum filtration and washed as described in Section 2.2.6a. Total radioactivity in a 100 μl sample was measured in duplicate by counting samples on pre-wet (to account for possible quenching) filters. Non-specific uptake was determined by incubation

of the synaptosomes with [^{3}H] DA at 4°C and sampling and washing at time zero (Schoemaker and Nickolson, 1983). Uptake was corrected for non-specific uptake and expressed as a percentage of the total radioactivity.

c) The effect of synaptosomal protein concentration on the uptake of [^{3}H]DA

The uptake of [^{3}H] DA (0.11 μM) by striatal synaptosomes was measured over the protein concentration range 0.2 - 4 mg/ml. A preparation of synaptosomes was diluted with perfusion medium and uptake determined at each protein concentration (as described in Section 2.2.6a), after a 5 min incubation period with the radiolabel. All assays were carried out in duplicate.

2.2.7 Radioactivity counting

a) Scintillation spectrometry

i) Comparison of the counting efficiency of different scintillants

The counting efficiency of different scintillants was determined by counting a 10 μl sample of [^{3}H] DA (0.1 μCi) in the presence of increasing amounts of perfusion medium and a constant volume of scintillant (5 ml; Fig. 2.2a). The scintillant 'Aqua Luma' was initially used because of its high counting efficiency over the required aqueous concentration range. In later experiments 'Optiphase Safe' was used because it was less expensive and of similar counting efficiency. A full range calibration curve for Optiphase Safe is shown in Fig. 2.2b. The presence of a minivial inside the main vial caused no additional quenching.

To ensure a high counting efficiency scintillant was added to aqueous samples to produce a 10% aqueous mixture.

ii) Fractions from perfusion experiments

To an 8 drop fraction (340 µl) 3 ml of Optiphase was added to give approximately 30% counting efficiency.

iii) Filters

Filters were placed in the bottom of a 20 ml plastic scintillation vial with 5 ml Optiphase Safe and counted after 24 h at 4°C by which time the counting efficiency had reached a maximum (36%).

All samples were counted for 2 min in a Packard TriCarb spectrometer using external standardisation.

b) Gamma-counting

Samples containing [^{125}I] were counted for 1 min an LKB 2280 Ultrogamma counter.

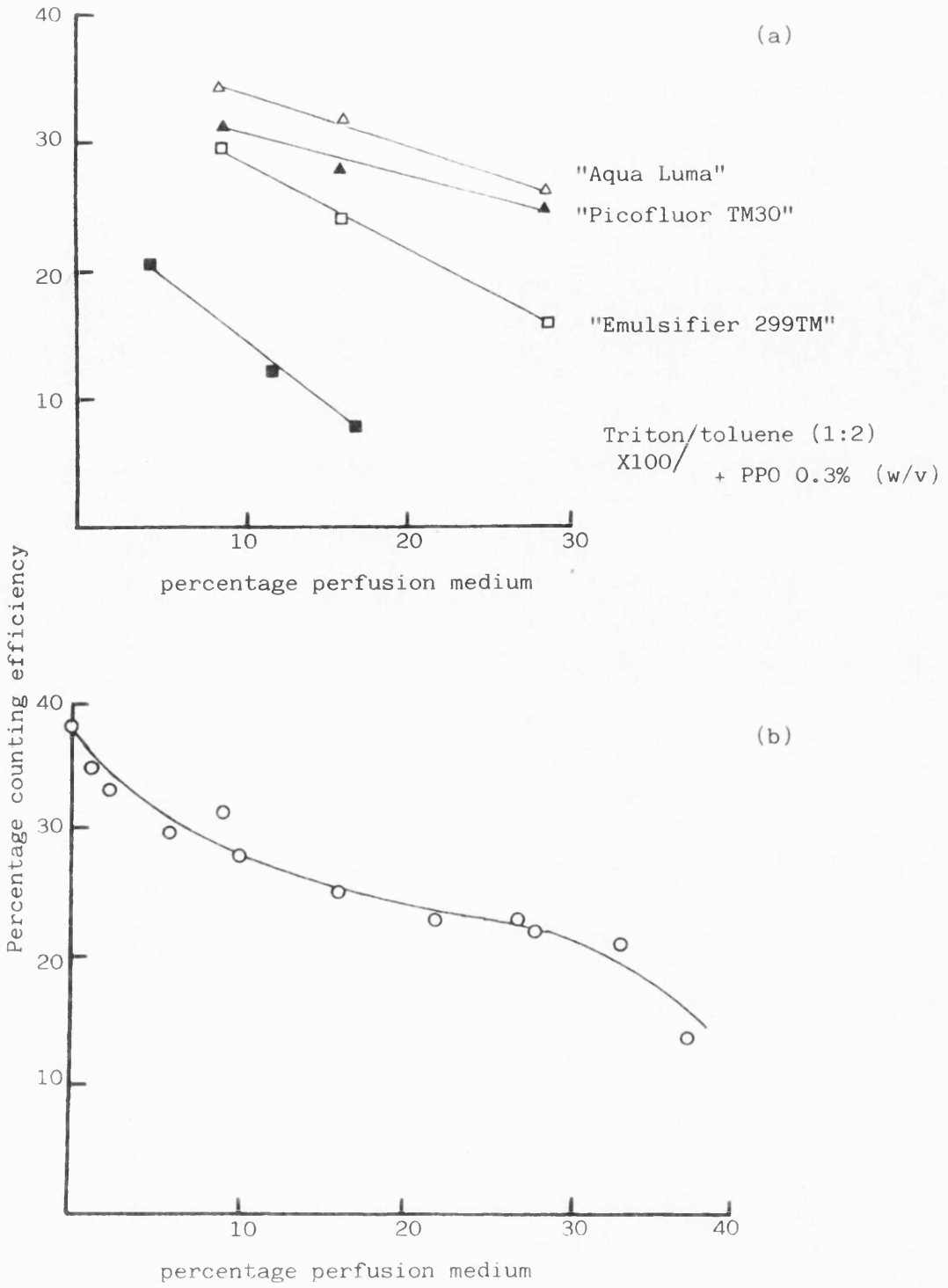


Fig. 2.2. Liquid scintillation counting efficiency

a) Counting efficiency of various scintillants in the presence of increasing amounts of aqueous medium.

b) Standard curve for "Optiphase Safe".

2.3 RESULTS

2.3.1 Preparation of synaptosomes and analysis of the gradient fractions by electron microscopy

The one-step centrifugation method used for isolating synaptosomes from a crude homogenate was very quick and simple. Within 1½ h after the death of the rats,a synaptosomal fraction was obtained which appeared as a distinct band in the sucrose gradient (Fig. 2.3).

Analysis of the 3 fractions (P_a, P_b, P_c; Fig. 2.3) under the electron microscope, showed the characteristic morphology of the structures present in each band. The P_a fraction consisted of electron dense concentric rings characteristic of myelin fragments with little contamination from other structures. The pellet (P_c) consisted of cell bodies (containing large nuclei) and cell debris in addition to free mitochondria. The size of the cell bodies ranged from 7 - 13 μm.

Under low magnification the P_b fraction consisted of numerous spherical structures, some identifiable as synaptosomes others as mitochondria. Analysis under high magnification (Fig. 2.4) using the two different staining techniques of positive and negative staining, showed in greater detail the morphology of the synaptosomes. Using negative staining (Fig. 2.4b) the synaptosome appeared as a thin-walled bag containing vesicles. For the positive staining two stains were compared; 1% tannic acid and 2% aqueous uranyl acetate. Both stains gave similar results although at high magnification the internal structures of the synaptosomes were more defined in sections stained with 1% tannic acid.The positively stained section revealed the intracellular components: mitochondria, vesicles and vacuoles. Also attached were a few dark regions on the plasma membrane, these

Fig. 2.3. Preparation of synaptosomes

Synaptosomes were prepared from a 10% (w/v) 0.32 M sucrose (pH 7.4) homogenate by discontinuous sucrose gradient centrifugation (10,000 *g* for 60 min at 4°C). The resulting gradient bands were labelled as shown opposite. Because the synaptosomal band at the 0.8 - 1.2 M sucrose interface had been prepared by a one step centrifugation process it was called P_b rather than the conventional labelling P_2b (Gray and Whittaker, 1962), where synaptosomes are isolated from the second pellet (P_2).

The 3 membrane fractions (P_a, P_b and P_c) were examined under the electron microscope (p.**63**). The micrographs showed clearly the morphology of the structures isolated in each band.

P_a Myelin fraction (stain = 1% tannic acid)

Myelin (my) was identified by its multilamellar structure.

P_b Synaptosomal fraction (stain = 1% tannic acid)

Numerous synaptosomal profiles (s) and a few isolated mitochondria (m) were characteristic of this fraction.

P_c Cell debris (stain = 2% aqueous uranyl acetate)

This fraction consisted of the cell bodies and unidentifiable fragments. Within the cell bodies were large, heavily stained nuclei (n).

Also present are mitochondria (m)

Bar represents 2 µm.

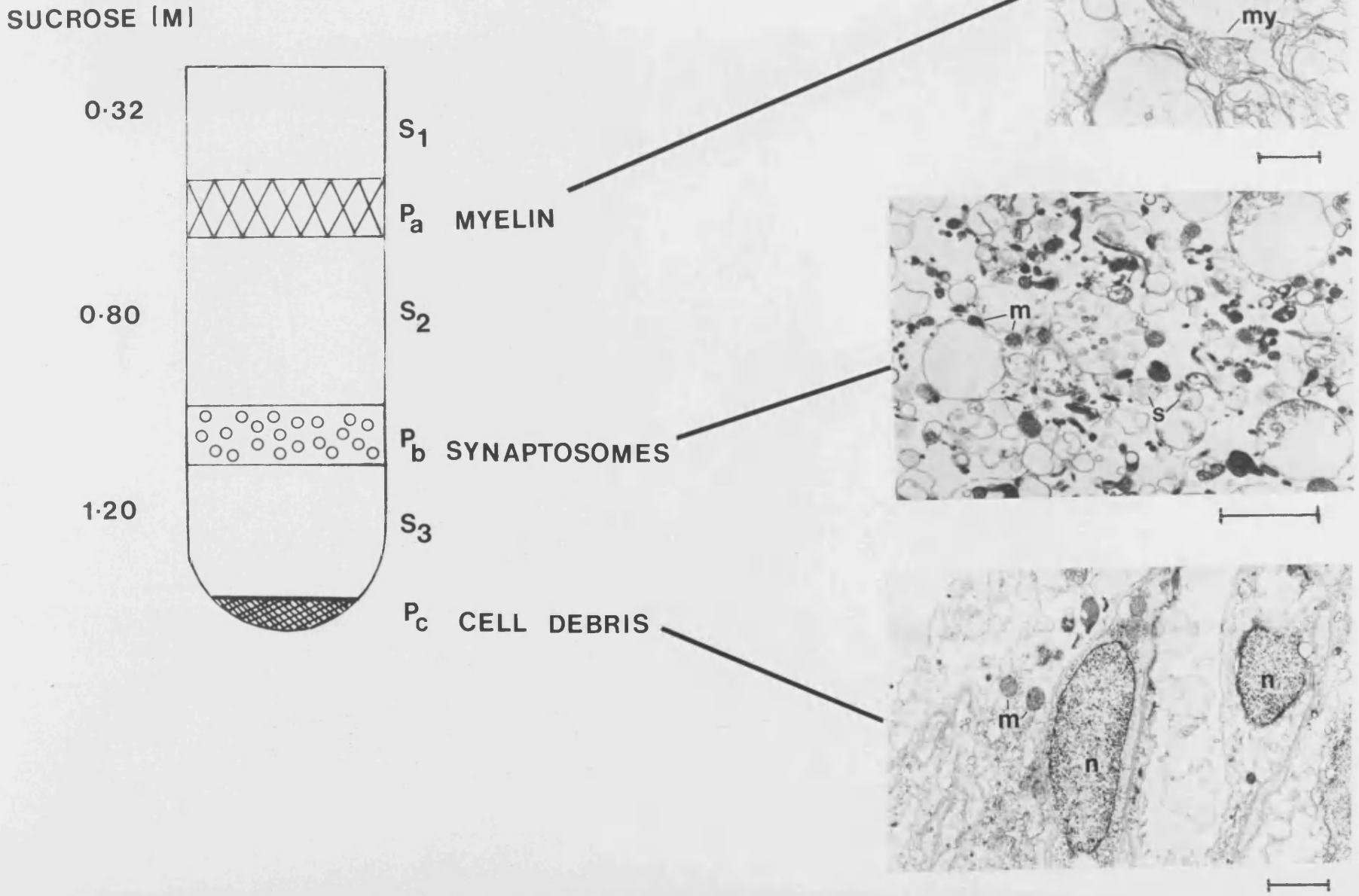
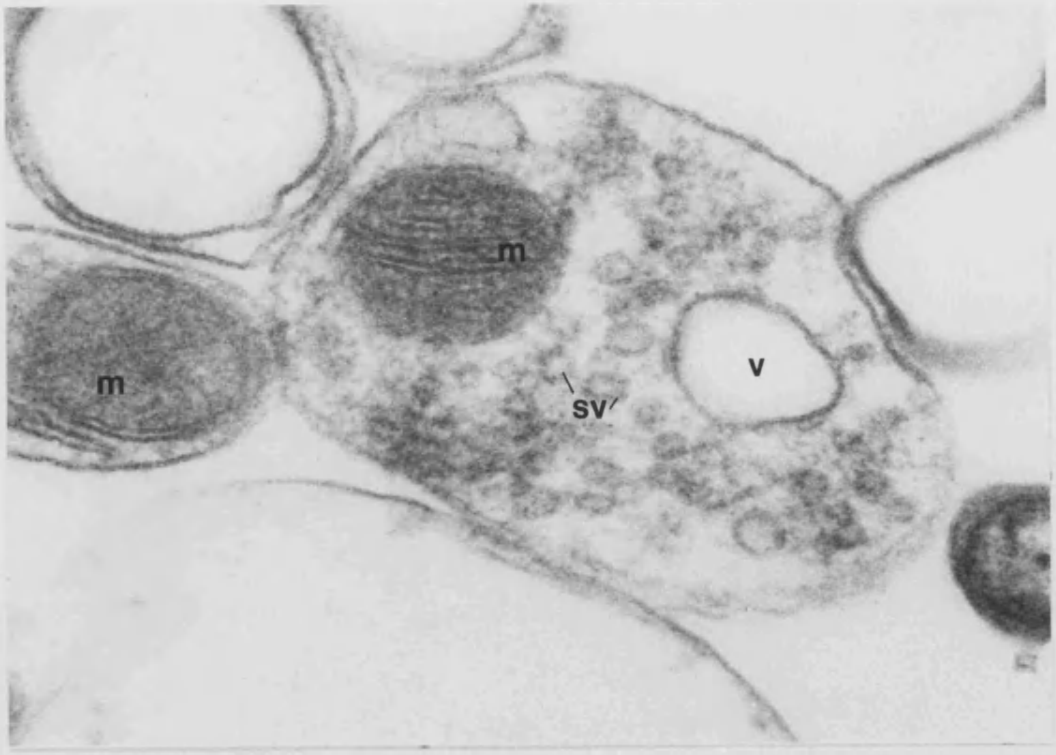

SUCROSE (M)
0·32
0·80
1·20
S1
Pa
S2
Pb
S3
Pc
MYELIN
SYNAPTOSOMES
CELL DEBRIS
my
m
s
m
n
n

Fig. 2.4. Striatal synaptosomes viewed under the electron microscope (high power magnification)

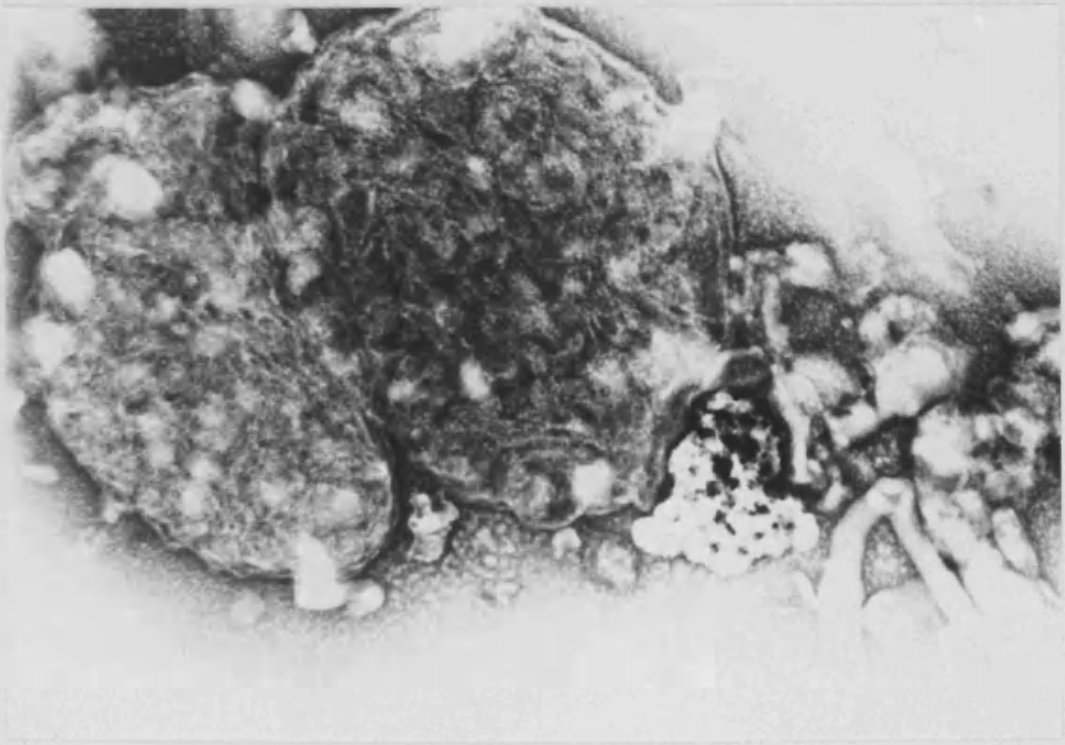
a) Positively stained synaptosomes (1% tannic acid) containing synaptic vesicles (sv), mitochondria (m) and a vacuole (v).

b) Negatively stained synaptosomes visualised as thin-walled bags.



a.

0·5 µm



b.

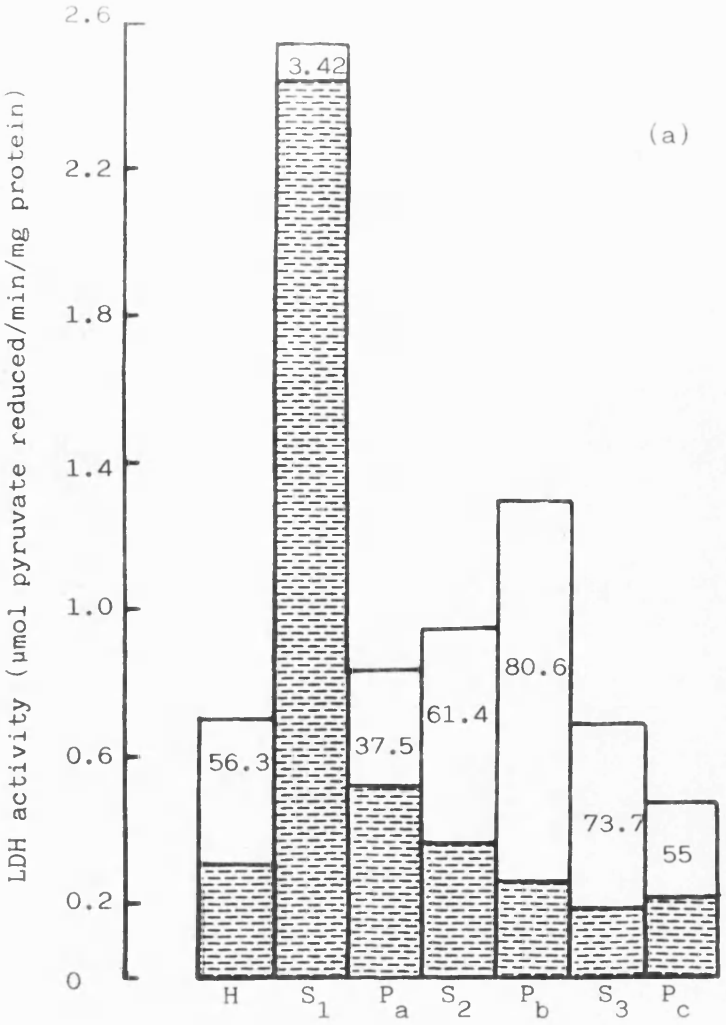
0·5µm

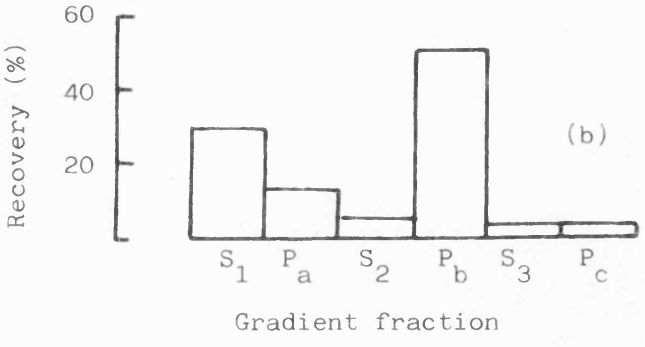
Fig. 2.5. Distribution of LDH activity in the gradient fraction.

a) LDH activity was measured in each gradient fraction. The free (shaded) and the total (unshaded) activities were determined in the absence and presence of Triton X-100, respectively. The occluded activity was calculated as the difference between the two activities and expressed as a percentage of the total value (numbers).

b) The recovery of LDH activity in each gradient fraction represented as a percentage of the total activity in the original homogenate.

Results from a representative experiment.


LDH activity (µmol pyruvate reduced/min/mg protein)
2.6
2.2
1.8
1.4
1.0
0.6
0.2
0
3.42
56.3
37.5
61.4
80.6
73.7
55
(a)
H
S1
Pa
S2
Pb
S3
Pc


Recovery (%)
60
40
20
(b)
S1
Pa
S2
Pb
S3
Pc
Gradient fraction

were identified as post synaptic thickenings. The approximate size of the synaptosomes was 0.6 - 1 μm.

2.3.2 Distribution of LDH activity in the gradient fractions

The cytoplasmic marker LDH was used to measure the integrity of the synaptosome preparation. LDH activity was distributed throughout the sucrose gradient (Fig. 2.5a). The highest percentage occluded activity was found in the P_b band, indicating an enrichment of sealed membraneous structures. The mean occluded LDH activity in this synaptosome band was 79.13 ± 4.88% of total activity (n = 8, mean ± SEM). Over 50% of the total LDH activity was recovered in the P_b fraction (Fig. 2.5b).

2.3.3 Binding of [^{125}I]α-BGT and [^{3}H]nicotine to rat brain membranes

a) The relationship of radioligand binding and protein concentration

The binding of [^{3}H]nicotine (40 nM) and [^{125}I]α-BGT (5 nM) to P_2 membranes was shown to be linear with respect to protein concentration (Fig. 2.6 a and b respectively). Subsequent binding assays were carried out using protein concentrations of approximately 2 mg/ml.

b) Analysis of radioligand binding by Scatchard analysis

The simplest model used to describe the ligand receptor interaction assumes that a single set of ligand molecules, L, reacts reversibly with a single set of receptor molecules, R, to form a ligand receptor complex LR,

$$L + R \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} LR \qquad (1)$$

At equilibrium

$$\frac{[L][R]}{[LR]} = \frac{k_{-1}}{k_{+1}} = K_d \qquad (2)$$

The constants k_{+1} and k_{-1} are the kinetic constants for association and dissociation respectively and K_d is the equilibrium dissociation constant.

Alternatively, the K_d is determined at equilibrium using the Scatchard equation (5). When R in equation 2 is equal to R_T - LR where R_T represents the total concentration of receptors, equation 2 can be rearranged:-

$$[R_T - LR]\ [L]\ /[LR] = K_d \qquad (3)$$

If LR is defined as the bound ligand (B), L as free ligand (F) and R_T as the maximum number of specific receptor sites (B_{max}), equation 3 becomes

$$(B_{max} - B)\ (F)\ /\ B = K_d \qquad (4)$$

which can be rearranged

$$B/F = \frac{(B_{max} - B)}{K_d} \qquad (5)$$

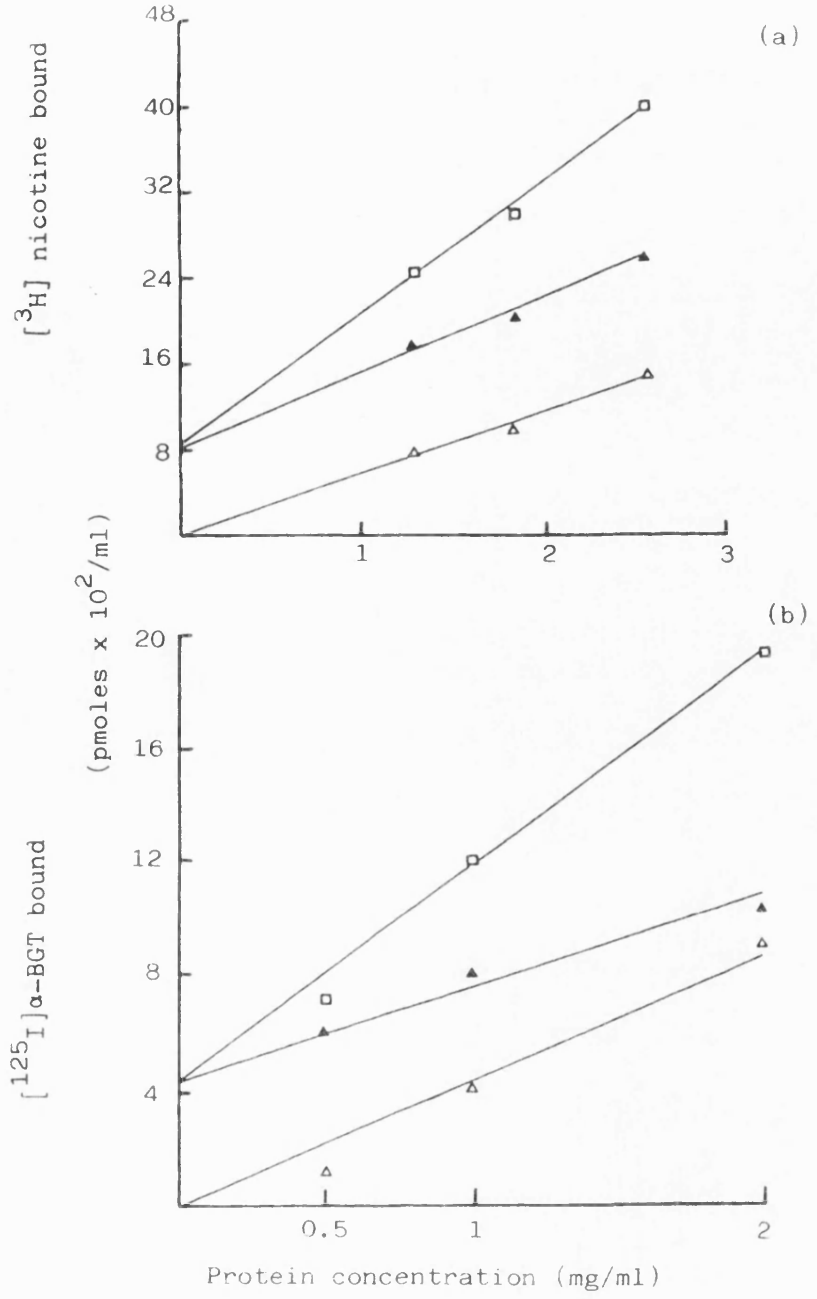


Fig. 2.6. Protein dependence of [^{3}H]nicotine and [^{125}I]α-BGT binding to P_2 membranes.

a) [^{3}H]nicotine (40 nM) and b) [^{125}I]α-BGT (5 nM) were incubated with varying amounts of membrane samples for 60 min at 22°C. Non specific binding was measured in the presence of 100 μM nicotine and 0.5 μM αBGT respectively.

(□) total binding, (▲)non-specific, (△) specific.

This is the Scatchard equation (Scatchard, 1949). A plot of B/F versus B gives either a straight line with a slope of K_d and an intercept with the abscissa of B_{max}, or a non-linear curve, indicating heterogeneity or cooperativity of the binding sites for the ligand studied.

c) Binding of [^{125}I] α-BGT to rat brain membranes

Specific binding of [^{125}I]α-BGT to rat brain membranes was measured over the concentration range 0.2 - 10 nM (Fig. 2.7). Scatchard analysis of the data gave a straight line with an apparent K_d value of 2.99 ± 0.25 nM and a B_{max} value of 67.3 ± 8.2 fmoles/mg protein for the binding to P_2 membranes. For the binding to striatal membranes K_d and B_{max} values of 3.33 ± 1.07 nM and 82.2 ± 22.3 fmoles/mg protein respectively were obtained. Results are expressed as the mean ± range of 2 experiments carried out on different membrane preparations.

d) Binding of [^{3}H]nicotine to rat brain membranes

Saturable specific binding of [^{3}H] (+/-) - nicotine to P_2 and striatal membranes was shown (Figs. 2.8a and b).

For P_2 membranes, Scatchard analysis of the binding data yielded a straight line (r^2 = 0.900) giving an apparent K_d of 16 nM and B_{max} value of 80 fmoles/mg protein, (single determination). These data suggest the presence of a single population of binding sites.

Because of the low amounts of striatal tissue available, initial binding studies were carried out using only 5 concentrations of [^{3}H] nicotine. Curvilinear Scatchard plots were obtained, suggesting either cooperativity or heterogeneity of binding sites. A more detailed study using 10 concentrations of [^{3}H] nicotine also resulted in a curvi-

linear Scatchard plot (Fig. 2.8b). The data obtained were analysed using the method of Humrich and Richardson (1983) modified for the BBC computer by B. McCormack (Bath University). A high affinity binding site with an apparent K_d of 1-5 nM and B_{max} of approximately 80 fmoles/mg protein identified.

e) Subcellular distribution of [^{125}I]α-BGT and [^{3}H]nicotine binding

Comparison of the binding of [^{125}I]α-BGT (5 nM) and [^{3}H] nicotine (40 nM) binding to the 3 gradient fractions (P_a, P_b and P_c; Fig. 2.3) clearly demonstrated that the synaptosomal fraction contained a higher concentration of both [^{125}I]α-BGT and [^{3}H]nicotine binding sites (Fig. 2.9). There was also appreciable binding to the cell debris (P_c) fraction.

2.3.4 Uptake of [^{3}H]DA by striatal synaptosomes

The uptake of [^{3}H]DA (0.05 - 1 μM) by striatal synaptosomes (protein concentration 1 mg/ml) was saturable and inhibited by nomifensine (50 μM; Fig. 2.10). The Michaelis constant (K_m) and the V_{max} for the specific uptake was determined by the direct linear plot (Eisenthal and Cornish-Bowden, 1974; Cornish-Bowden and Eisenthal, 1978). Values of 0.24 ± 0.03 μM for the K_m and 91.4 ± 9.5 pmoles [^{3}H] DA/mg protein/min for the V_{max} were obtained. Results are expressed as the mean ± standard error.

Uptake of [^{3}H]DA (0.11 μM) was shown to be linear for the first 2 min (Fig. 2.11), reaching a maximum at 5 min. From this experiment an uptake value of 40 - 50 pmoles [^{3}H]DA/mg protein was obtained. The uptake was also shown to be proportional to the amount of protein;

Fig. 2.7. Saturability of [^{125}I]α-BGT binding.

P_2 membranes (a) and striatal synaptosomal membranes b) were incubated for 60 min at 22°C with increasing concentrations of [^{125}I]α-BGT in the presence (0) or absence (●) of 0.5 μM α-BGT. Inset represents a Scatchard plot of the specific binding (Δ) from which the apparent K_d and B_{max} were calculated.

(a) K_d = 2.64 nM, B_{max} 75.3 fmoles/mg protein
(r^2 = 0.822)

(b) K_d = 2.26 nM, B_{max} 59.9 fmoles/mg protein
(r^2 = 0.815)

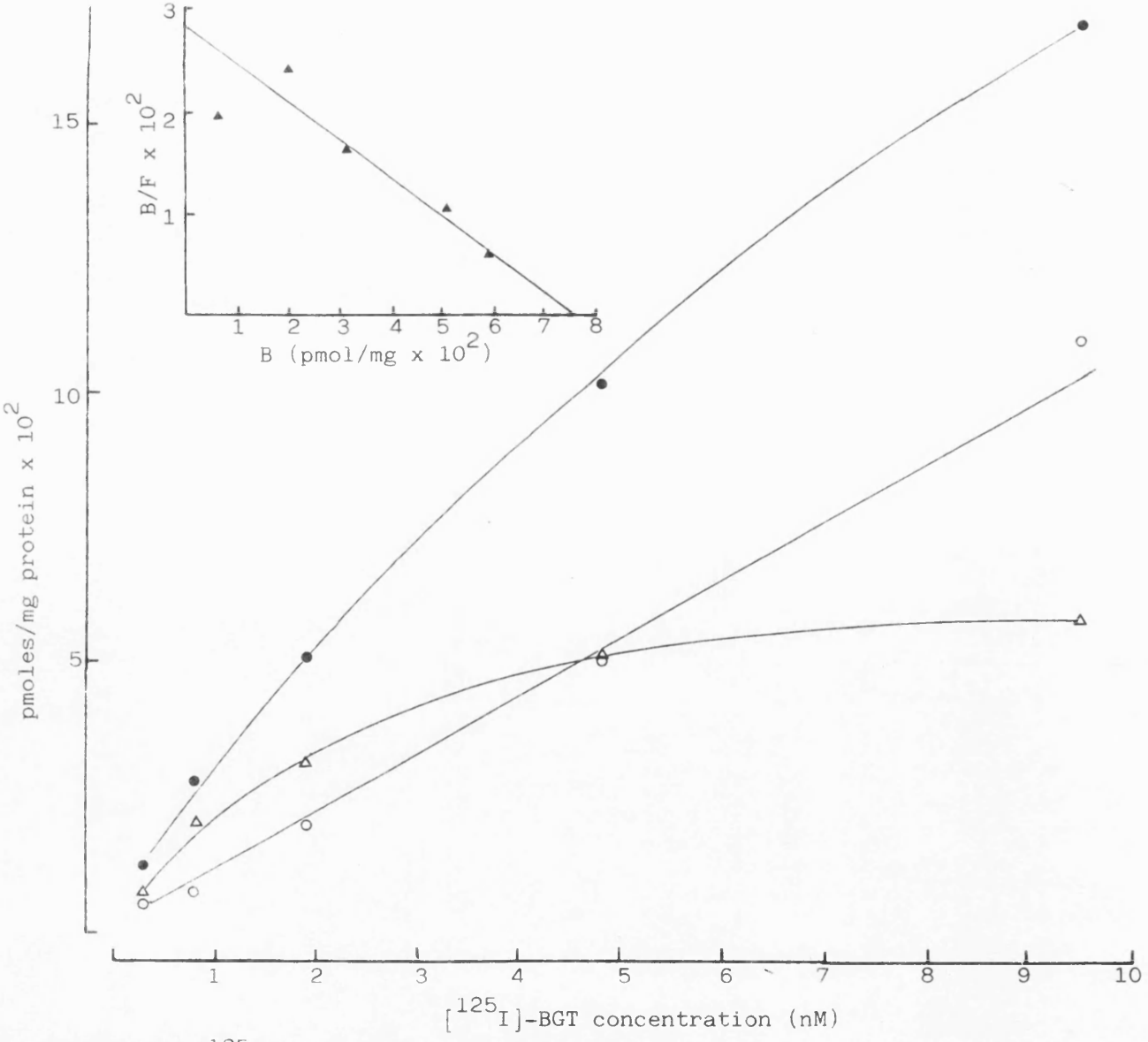


Fig. 2.7a. Binding of [^{125}I]α-BGT to rat brain P_2 membranes.

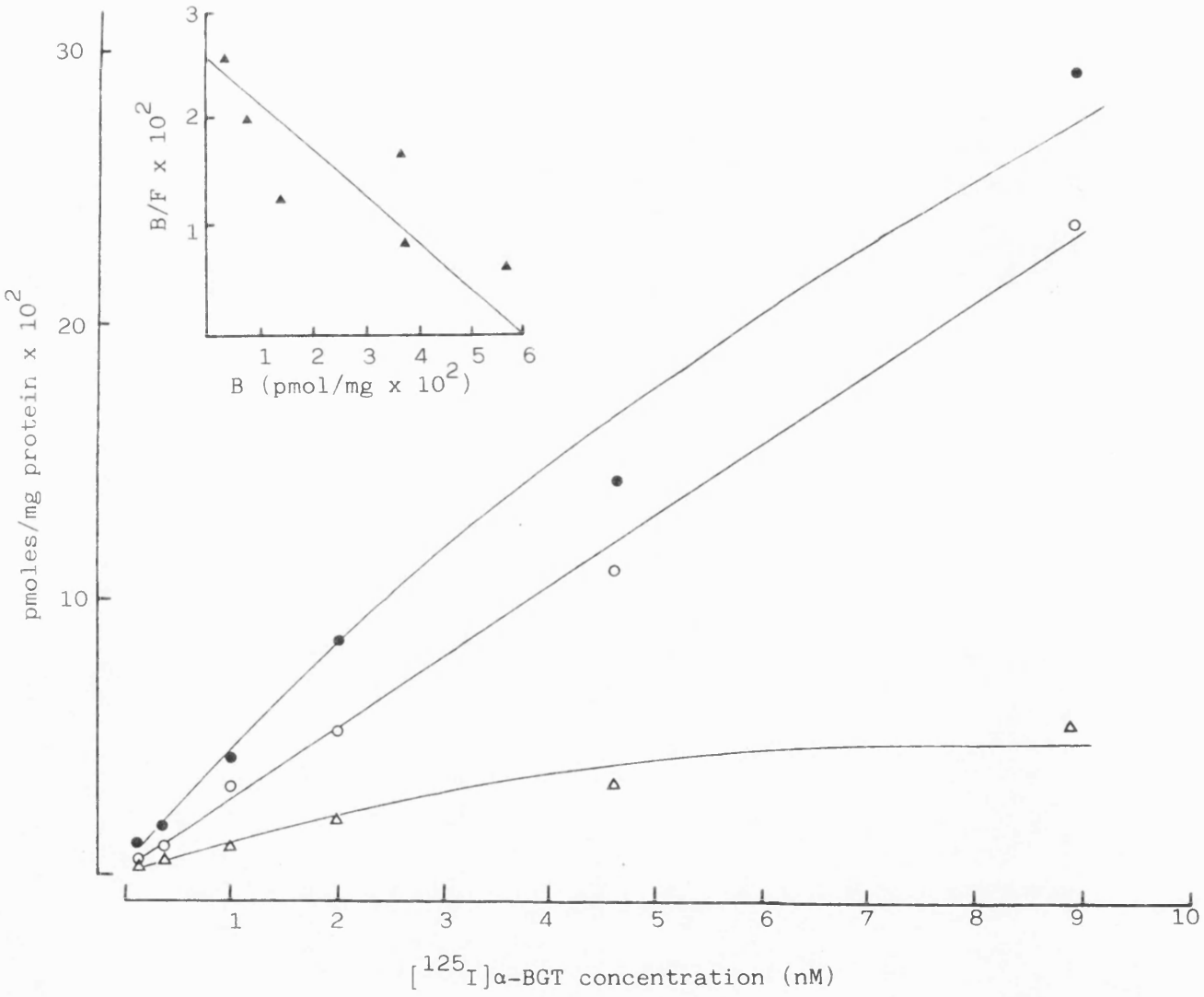


Fig. 2.7b. Binding of [^{125}I]α-BGT to striatal synaptosomal membranes

Fig. 2.8. Specific binding (●) of [^{3}H](+/-)-nicotine to a) P_2 membranes and b) striatal synaptosomal membranes was determined by incubation with increasing concentrations of [^{3}H](+/-)-nicotine in the presence and absence of 100 µM (-)-nicotine. Scatchard analysis of the data (lower plots) yielded the following binding constants:

a) Straight line Scatchard (r^2 = 0.900)

K_d = 16 nM, B_{max} = 80 fmoles/mg protein

b) Curvilinear plot analysed by the method of Humrich and Richardson (1983), modified by B. McCormack (Bath University) for the BBC computer.

High affinity site K_d=1-5 nM B_{max} ≅ 80 fmoles/mg protein.

Low affinity site, unsaturable.

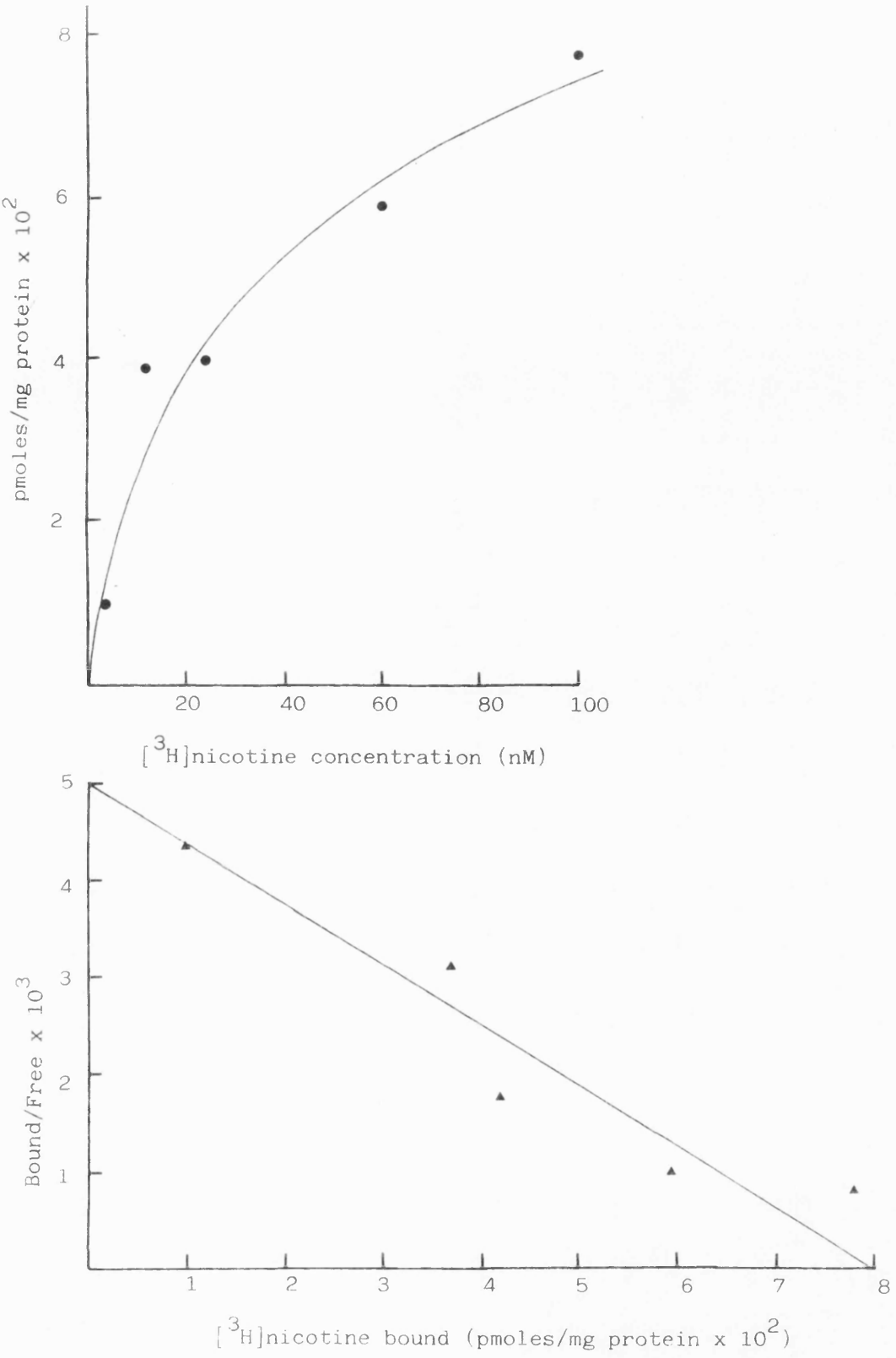


Fig. 2.8a. Specific binding of [^{3}H]nicotine to P_2 membranes.

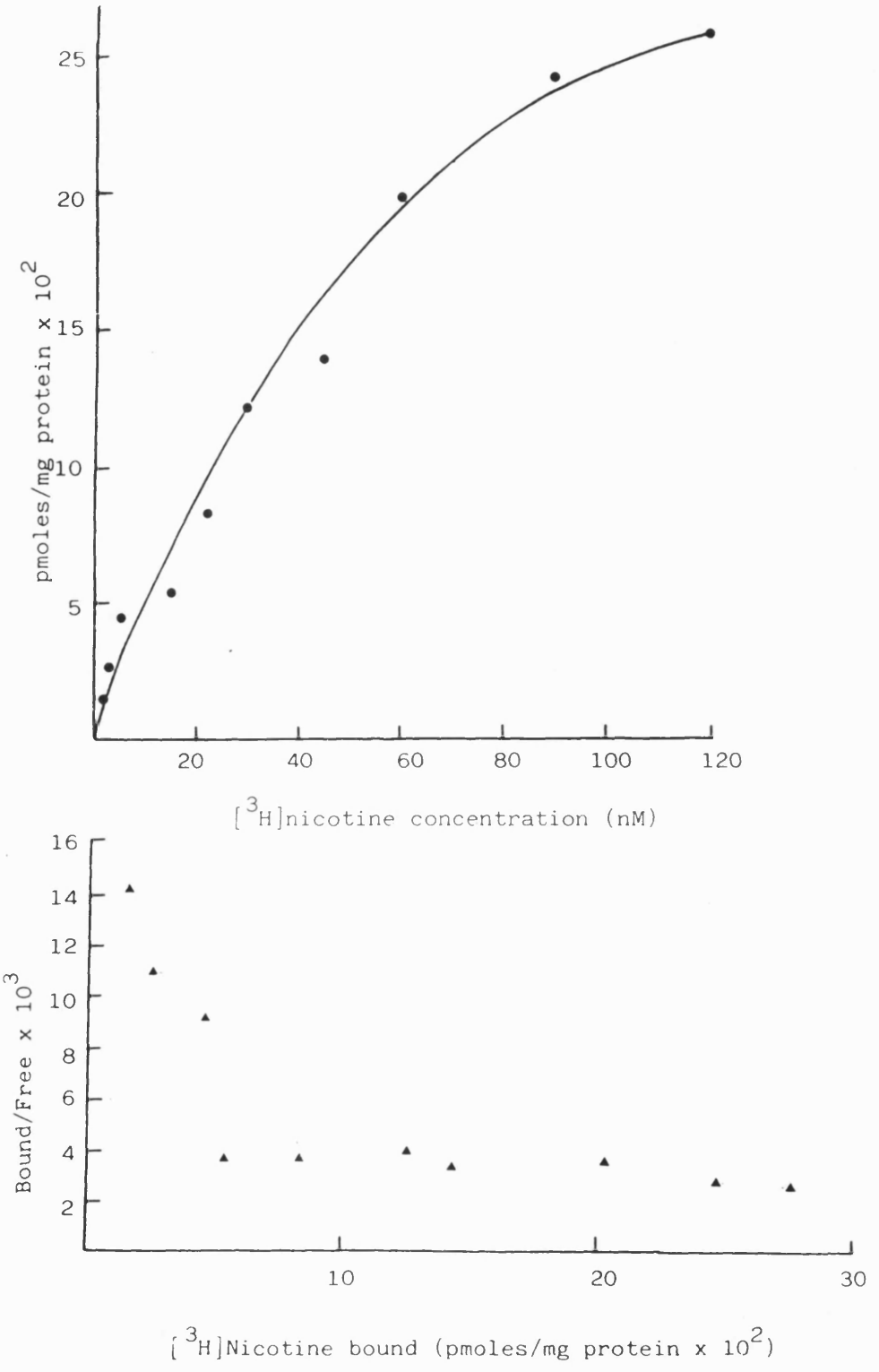


Fig. 2.8b. Specific binding of [^{3}H]nicotine to striatal synaptosomal membranes.

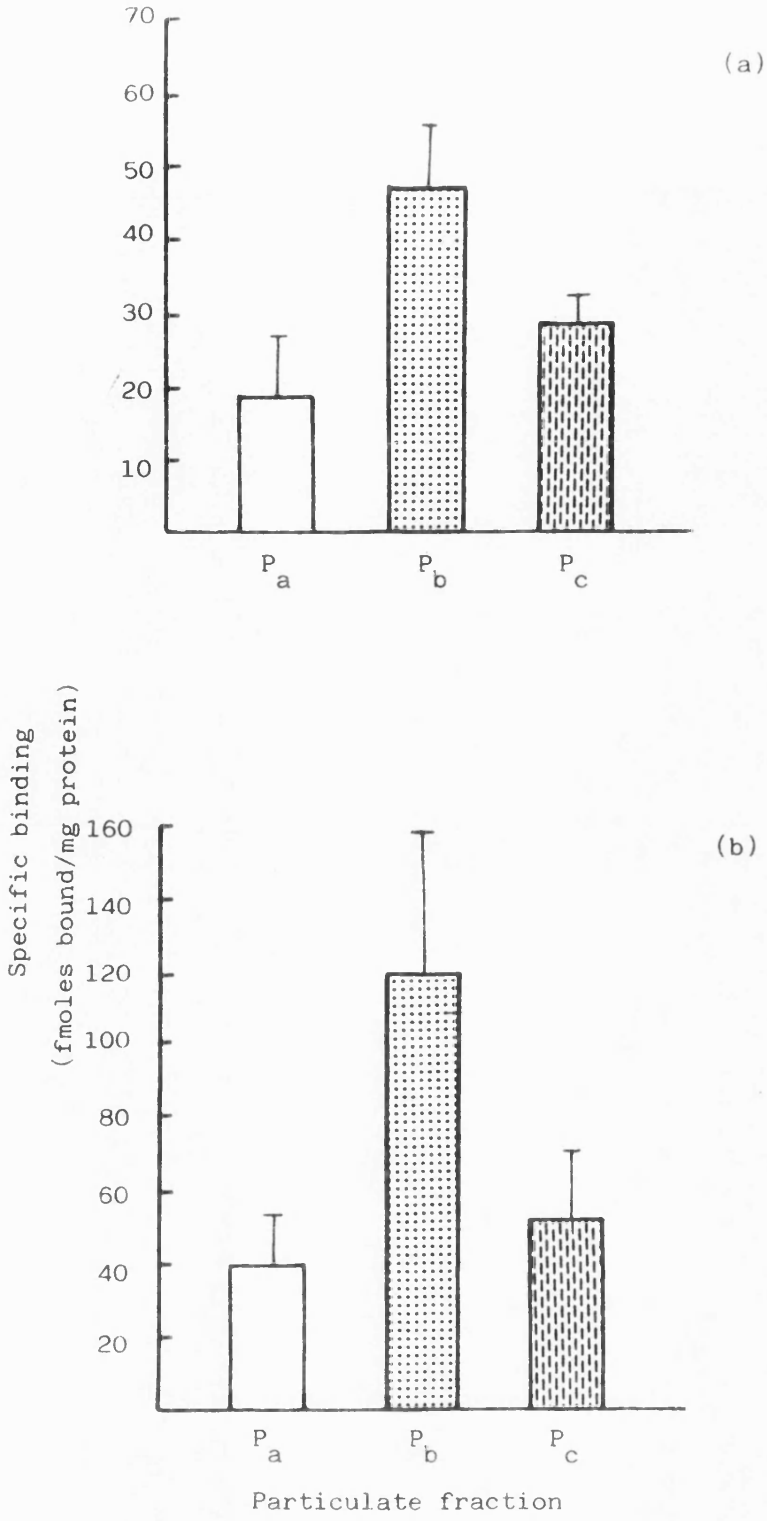


Fig. 2.9 Subcellular distribution of the binding of (a) [^{125}I]α-BGT (5 nM) and (b) [^{3}H]nicotine (40 nM). Specific binding was determined as described on p.70 . Results are expressed as the mean of two experiments. Error bars represent the range of results.

optimal loading being achieved at a protein concentration of 2 mg/ml (Fig. 2.12).

The Na^+- dependency of the uptake was demonstrated by incubation in Na^+-depleted medium (p.137). At a high DA concentration of 10 µM only 67% of the normal uptake was observed in Na^+-depleted medium. In the presence of nomifensine (50 µM) 34% of the normal uptake of [^{3}H]DA (10 µM) occurred. The uptake was also temperature sensitive; at 4°C uptake of [^{3}H]DA (10 µM) was 17% of control (37°C) and at a lower [^{3}H] DA concentration (0.11 µM) only 5% of the control value.

2.3.5 Conditions used to preload the striatal synaptosomes with [^{3}H]DA

To achieve maximum loading of the synaptosomes with [^{3}H]DA, synaptosomes were resuspended in perfusion medium to give a protein concentration of 2 - 4 mg/ml. After a 10 min preincubation period at 37°C, [^{3}H]DA was added, final concentration 0.11 µM, (10 µl of stock (11 µM) per ml synaptosomes). Since the data shown in Fig. 2.11 indicate that maximum uptake is achieved in only 5 min and thereafter a gradual decline occurs, incubation with [^{3}H]DA was continued for 5 min.

2.3.6 The effect of washing by centrifugation on synaptosomal integrity

A method for removing [^{3}H]DA which had not been taken up by the striatal synaptosomes was required to reduce the washout period in the perfusion experiments (p.117). The effect of washing the preloaded synaptosomes by centrifugation was determined as described below.

Fig. 2.10. Uptake of DA by striatal synaptosomes

a) After 10 min preincubation (37°C) samples of striatal synaptosomes (1 mg protein/ml) were incubated with a range of DA concentrations. Uptake was determined by filtration (▲), non-specific uptake was measured in the presence of nomifensine (50 µM, △).

b) Specific uptake was analysed using the direct linear plot. A K_m value of 0.24 µM (standard error ± 0.03) and a V_{max} of 91.4 ± 9.5 pmoles/mg protein/min were obtained.

Results are from a single experiment, determinations carried out in duplicate.

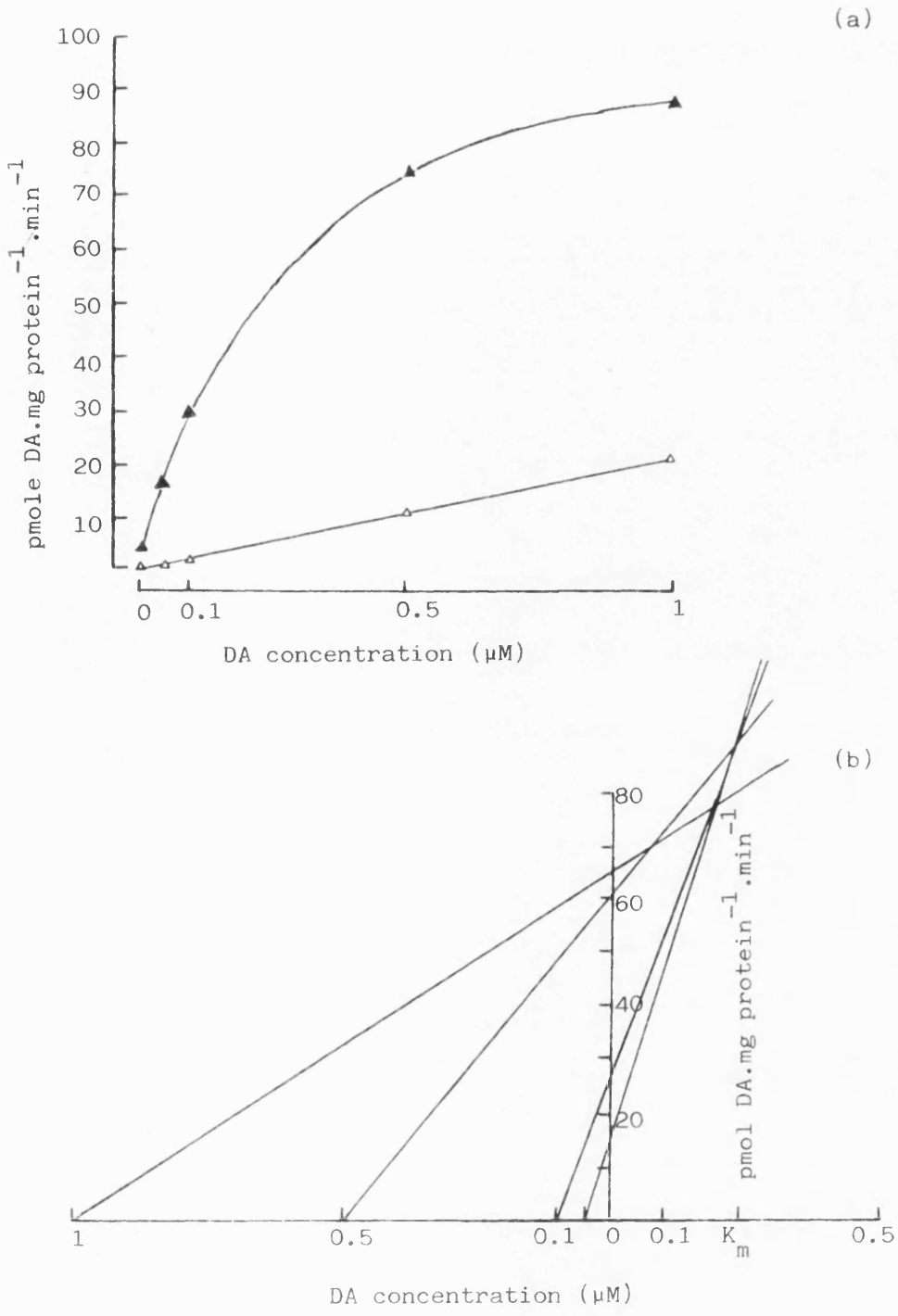


Fig. 2.10. Uptake of DA by striatal synaptosomes

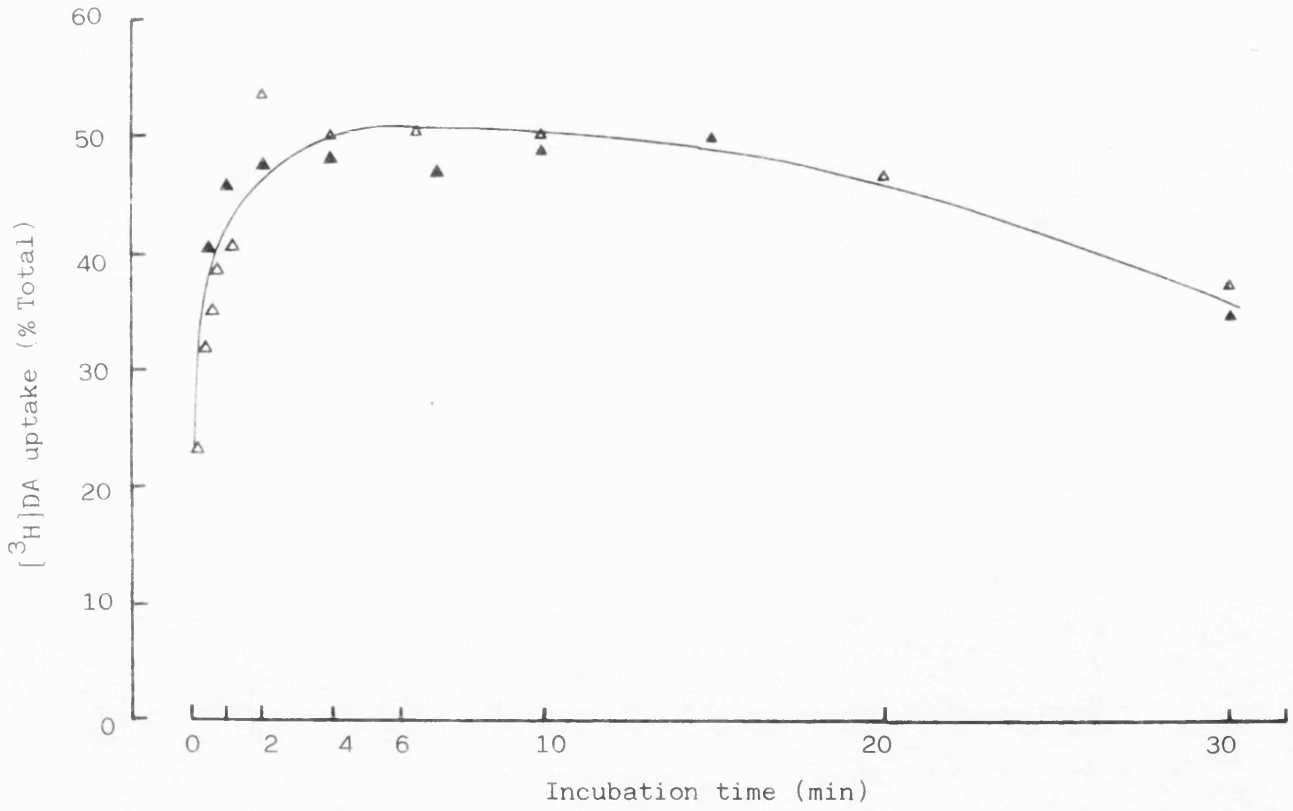
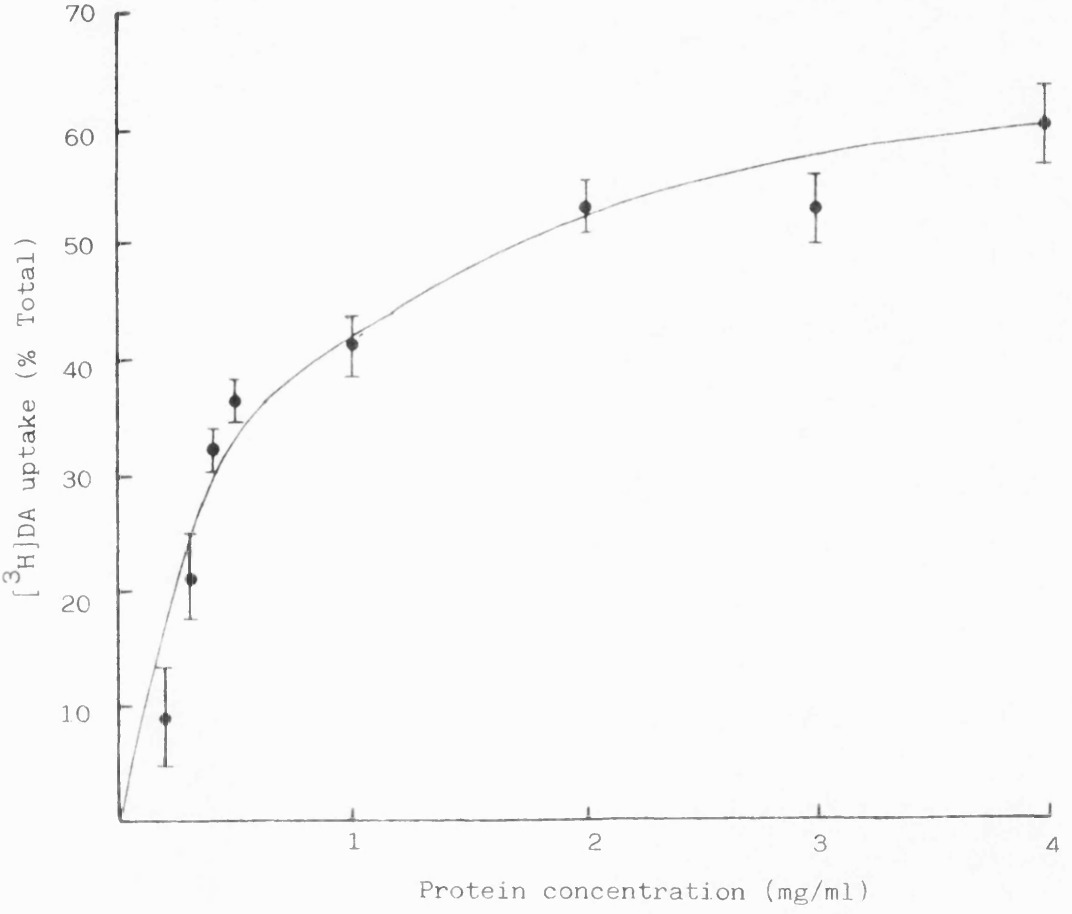


Fig. 2.11. Time course of [^{3}H]DA uptake into striatal synaptosomes

After 10 min preincubation, (37°C),[^{3}H]DA (0.11 µM) was added to a suspension of synaptosomes. At time intervals, a 100 µl sample was withdrawn and uptake determined (p.**72**). Results are the combination of two sets of data (△ , ▲) using different preparations.

Fig. 2.12. Effect of protein concentration on the uptake of [^{3}H]DA by striatal synaptosomes.

A preparation of synaptosomes was diluted with perfusion medium and for each protein concentration uptake of [^{3}H]DA (0.11 µM) was determined as described on p.71 , after incubation at 37°C for 5 min with the radiolabel. Results represent the mean ± range of 2 determinations (2 preparations).



Striatal synaptosomes were prepared and loaded with [^{3}H]DA using the optimum conditions (Section 2.3.5). The uptake of [^{3}H]DA was determined as outlined on p.**71** and the integrity of the preparation was determined by measuring the occluded LDH activity (p.**67**). The remaining suspension was centrifuged at 1,000 g for 7 min at 4°C and the resulting pellet was gently resuspended in medium to its original concentration. Further samples were removed for uptake and LDH determinations and the washing procedure was repeated. Throughout the washing steps the synaptosomes were kept at 4°C to prevent further [^{3}H]DA uptake.

Table 2.1. The effect of washing synaptosomes by centrifugation.

Number of washes	[^{3}H]DA uptake(%)	Occluded LDH activity	
		moles/min/mg protein	% total
0	54	1.04	81
1	82	1.02	91
2	85	1.00	92

The table above summarises the results obtained from a synaptosome preparation of protein concentration 2.75 mg/ml. The occluded LDH activity was retained throughout the successive washes, indicating that the synaptosomes remain intact and are not damaged by this washing procedure.

2.4 DISCUSSION

2.4.1 The synaptosomal preparation

The one-step centrifugation process was a quick and effective method for separating cell bodies, myelin and synaptosomes from a crude striatal homogenate, as revealed by observation of the gradient fractions under the electron microscope (Fig. 2.3). Using low power magnification the size of the cell bodies present in the P_2 fraction (7 - 13 μm) agrees with the reported size of medium sized neurones (which are the main type of neurone in the striatum) of 10 - 20 μm in diameter (Heimer *et al.*, 1985). The size of the synaptosomal fraction (P_b), viewed under high power magnification, of 0.6 - 1 μm also agrees with the reported range in size of rat brain synaptosomes of about 0.5 - 2 μm (Jones, 1975; Whittaker, 1984). The synaptosomal preparation was visualised under the electron microscope using both positive and negative staining techniques. However, although negative staining is much quicker than positive staining and is therefore a useful technique to analyse the integrity of a synaptosomal preparation on the same day as its preparation, negative staining gives little information about the internal structures present within synaptosomes (see Fig. 2.4). In contrast, using positive staining techniques, the internal organelles were clearly observed, an important characteristic of synaptosomes (Whittaker, 1984).

Measurement of the distribution of the occluded LDH activity in the sucrose density gradient is also a useful method for identifying the synaptosomal band. The analysis is carried out on the same day of preparation and can therefore be used to ensure that intact synaptosomes have been isolated before further studies are

carried out using the synaptosomal preparation. The high level of occluded LDH activity in the P_b fraction (Fig. 2.5) indicates the presence of sealed membranous structures and the result that over 50% of the total LDH activity was found in the P_b fraction shows that it is particularly rich in synaptosomal structures. Measurement of the occluded LDH activity also proved useful to ascertain the integrity of a synaptosomal preparation after repeated washing (Table 2.1). There was very little loss in the amount of occluded LDH activity after 2 centrifugational washes, which suggests that synaptosomes are stable structures, an important requirement for their use in perfusion experiments.

2.4.2 The uptake of [^{3}H]DA by striatal synaptosomes

The striatal synaptosomes prepared by the one-step centrifugational method displayed high affinity uptake of [^{3}H]DA similar to that described by Holz and Coyle (1974). The K_m value obtained of 0.24 µM agrees with previous reports, values ranging from 0.13 - 0.4 µM (Holz and Coyle, 1974; Synder and Coyle, 1969), the variation probably reflecting differences in the assay conditions employed (Copper and Carlson, 1983). Using a purified synaptosomal preparation Holz and Coyle (1974) obtained a V_{max} value of 125 pmoles DA/mg protein/min, which is slightly higher than the V_{max} reported in this thesis of 91 pmoles DA/mg protein/min (Section 2.3.4). This would suggest that the synaptosomes prepared using the one-step method are of a slightly lower purity than those prepared by the much longer two-step centrifugational procedure described by Holz and Coyle (1974).

Two methods for determining the non-specific uptake of [^{3}H]DA were used: incubation with the specific uptake inhibitor nomifensine (50 µM) and incubation at 4°C. In the presence of nomifensine more uptake occurred than at 4°C. This was particularly noticeable at high DA concentrations (10 µM) where the nomifensine blocked 67% of the total uptake whereas 83% of the total uptake was prevented by incubation at 4°C. At lower DA concentrations the difference between non-specific uptake measured by inclusion of nomifensine and incubation at 4°C was less marked. At 0.1 µM [^{3}H]DA 93% of uptake was blocked by 50 µM nomifensine and 95% by incubation at 4°C. These results suggest a component of the non-specific uptake which is insensitive to nomifensine and may represent binding to DA receptors.

The Na^+-dependency of the uptake was determined only at one concentration of [^{3}H]DA (10 µM). Complete Na^+-deficiency was not possible because the buffering nature of the perfusion medium relies upon $NaHCO_3/KH_2PO_4/CO_2$. Using a medium containing low Na^+ (24.9 mM) the uptake of DA was reduced by a third, which agrees with the work of Holz and Coyle (1974) who demonstrated that a reduction in the concentration of Na^+ caused a decrease in V_{max}.

To preload the striatal synaptosomes with [^{3}H]DA for the perfusion experiments described in Chapters 3 and 4, a concentration of 0.11 µM was chosen because it was close to the estimated K_d value and also at a concentration similar to that used by other groups (e.g. Schoemaker and Nickolson, 1983). The optimum incubation period of the radiolabel with the synaptosomes (2 - 4 mg protein, as defined in Section 2.3.5) of 5 min agrees with the work of Schoemaker and Nickolson (1983) and Bonnet *et al.* (1984). This

is in contrast to the much longer incubation periods often used (e.g. Mills and Wonnacott, 1984).

The subcellular fractionation procedure described in this thesis therefore produces synaptosomes which show the characteristic high affinity DA uptake of striatal dopaminergic nerve terminals. These results together with the electron micrographs and LDH activities provide evidence in support of the use of the one-step method for isolating synaptosomes.

2.4.3 The binding of [^{3}H]nicotine and [^{125}I]α-BGT to rat brain membranes

Initial experiments using whole rat brain membranes were carried out to reproduce published data. Specific binding of [^{3}H] nicotine and [^{125}I]α-BGT to rat brain membranes was demonstrated. The straight line Scatchard plot obtained for the binding of [^{3}H] nicotine to whole rat brain P_2 membranes is consistent with a single population of binding sites. The apparent K_d value obtained of 16 nM agrees with the reported K_d values for a high affinity [^{3}H] nicotine binding site in rodent brain (Romano and Goldstein, 1980; Marks and Collins, 1982; Costa and Murphy, 1983). The binding of [^{125}I]α-BGT to P_2 membranes was also saturable and the apparent K_d of 2.64 nM is in agreement with previous reports (see Schmidt *et al.*, 1980).

In contrast to the straight line Scatchard plot for the binding of [^{3}H]nicotine to P_2 membranes, a curvilinear plot was obtained for the binding to striatal synaptosomal membranes (Fig. 2.8b). A concave curvilinear plot is indicative of either negative cooperativity or multiple binding sites. A similar plot was

obtained by Yoshida and Imura (1979), who also reported high and low affinity [^{3}H]nicotine binding sites on whole brain synaptic membranes. However, there are few reports of saturation [^{3}H] nicotine binding studies using striatal tissue. Recently, Jenner *et al.* (1986) reported a high affinity [^{3}H]nicotine binding site in the striatum with an apparent K_d of 7.2 nM and B_{max} of 135 fmoles/mg protein. Also in agreement with the results reported in this thesis was the presence of a low affinity site which did not saturate (Jenner *et al.*, 1986). The specificity of the low affinity site has often been questioned (Marks and Collins, 1982) and recently Lippiello and Fernandes (1986) reported that low affinity [^{3}H]nicotine binding sites are not observed when protease inhibitors are present during the preparation of the membranes. This would explain why only high affinity [^{3}H]nicotine binding sites were observed for P_2 membranes (because EDTA and PMSF were present throughout the membrane purification procedure) whereas high and low [^{3}H]nicotine binding sites were found using striatal membranes (which were prepared in the absence of protease inhibitors). It is therefore not possible to measure the low affinity [^{3}H]nicotine binding site using the assay described. Nevertheless, the ability of nicotinic agonists to displace bound [^{125}I]α-BGT provides evidence for a low affinity nicotine binding site that may correspond to [^{125}I]α-BGT binding sites (see Wonnacott, 1986).

The observed differences in K_d values of high affinity [^{3}H] nicotine binding to striatal membranes (1-5nM) and whole rat brain P_2 membranes (16 nM) may be a result of competition by "endogenous ligands" present at different concentrations in the membrane preparations. Inhibition of both [^{125}I]α-BGT binding (Quik , 1982)

and [^{3}H] nicotine binding (Shershen *et al.*, 1984; Perry *et al.*, 1986) to rat brain membranes by endogenous factors in brain homogenates have been demonstrated. During the preparation of the P_b striatal membranes the tissue is washed by the density gradient centrifugation procedure and because of the low availability of striatal tissue the striatal membranes were four times more dilute than the P_2 membranes. These differences would account for lower amounts of endogenous ligands and hence the lower K_d value for [^{3}H] nicotine binding observed using striatal tissue. This possibility has recently been verified in the laboratory by Wonnacott and MacAllan (personal communication) who showed that an additional wash of the P_2 membranes, before determination of [^{3}H]nicotine binding, greatly reduced the K_d value. In contrast to the difference observed in the K_d values of [^{3}H]nicotine binding to P_2 membranes and striatal membranes the K_d values for [^{125}I]α-BGT binding were similar. This suggests that any endogenous ligands present are possibly specific for the high affinity [^{3}H]nicotine binding site rather than for the [^{125}I]α-BGT binding site.

There are reports that the binding of α-BGT to the striatum is low compared with other brain regions (Marchand *et al.*, 1979) whereas regional [^{3}H]nicotine binding studies have shown that the striatum contains a high number of high affinity [^{3}H]nicotine binding sites compared with other regions (Jenner *et al.*, 1986). These regional distribution binding studies agree with the auto-radiographical studies of Clarke *et al.* (1985a)and indicate that the striatum is rich in high affinity [^{3}H]nicotine binding sites and low in [^{125}I]α-BGT binding sites. Comparison of [^{125}I] α -BGT and [^{3}H]nicotine binding to the striatal synaptosomal fraction

(Fig. 2.9) shows that there are almost twice as many high affinity [^{3}H]nicotine binding sites as there are [^{125}I]α-BGT sites. However, the concentration of [^{3}H]nicotine used (40 nM, after Romano and Goldstein, 1980) was at a saturating concentration whereas the concentration of [^{125}I]α-BGT used (5 nM, after Schmidt, 1977) was at a value close to the estimated K_d value. The observed number of [^{125}I]α-BGT binding sites may therefore be an underestimate. However, B_{max} values for the binding of cholinergic ligands to whole brain and striatal membranes are not directly comparable (Section 2.3.3 c and d) because of differences in the purity of the two membrane preparations.

Comparison of the binding of [^{125}I]α-BGT (5 nM) and [^{3}H] nicotine (40 nM) to the three gradient fractions (P_a, P_b and P_c) demonstrated that the striatal synaptosomal fraction (P_b) contained a higher number of both [^{125}I]α-BGT and [^{3}H]nicotine binding sites (Fig. 2.9). This enrichment of [^{125}I]α-BGT binding in the synaptosomal fraction is consistent with previous reports (Salvaterra and Moore, 1973; Etervić and Bennet, 1974; Tindall *et al.*, 1978) and also with the reported binding of [^{125}I]α-BGT in synaptic regions in rat brain (Hunt and Schmidt, 1978) and the localisation of α-BGT conjugated to horseradish peroxidase at synaptic regions in rat brain (Lenz and Chester, 1977). Present on the synaptosomes will be attached a portion of the post-synaptic membrane as shown by De Robertis *et al.*, (1962). The binding of the radioligands may therefore be to either presynaptic, postsynaptic or to both membranes. Evidence for a presynaptic location of [^{125}I]α-BGT binding has been provided by de Belleroche and Bradford (1979) by the demonstration that destruction of the nigro-striatal pathway

with 6-hydroxydopamine reduces the number of [^{125}I]α-BGT binding sites in the striatum by half.

The enrichment of [^{3}H]nicotine binding to a synaptosomal preparation has also previously been demonstrated (Yoshida and Imura, 1979; Benwell and Balfour, 1985) and the binding of [^{3}H] nicotine specifically to dopaminergic nerve terminals in the striatum has been shown using autoradio-graphic and lesioning studies (Clarke and Pert, 1985). Using these techniques approximately a third of the high affinity [^{3}H]nicotine binding sites in the striatum were shown to be linked to dopaminergic nerve endings (Clarke and Pert, 1985), providing substantial evidence for the possible involvement of nAChR in the regulation of DA release.

CHAPTER 3

OPTIMISATION OF THE SYNAPTOSOMAL PERFUSION SYSTEM

3.1 INTRODUCTION

Many perfusion systems employing slice preparations have been described (e.g. Giorguieff *et al.*, 1976) however, relatively little work has been carried out using synaptosomes. The first synaptosomal perfusion systems were described in the early 1970's (de Belleroche and Bradford, 1972; Levy *et al.*, 1973; Raiteri *et al.*, 1974; Mulder *et al.*, 1975). The common basis for such procedures was a preloading of synaptosomes with radiolabelled transmitter or precursor followed by perfusion while the synaptosomes were retained on an inert support. One such systems was described in 1972 by de Belleroche and Bradford in which the synaptosomes were sandwiched in a nylon gauze immersed in buffer and additions made to and samples taken from, the surrounding medium. However, an important prerequisite of a perfusion system is the prevention of reuptake and this system did not completely satisfy this requirement. An improved procedure was described by Levy *et al.* (1973), which used a fibre-glass filter in a Swinex filter unit, the synaptosomes were retained while buffer flowed through the filter. A variation was described by Raiteri *et al.*, (1974), in which the support was a nitrocellulose filter (Millipore, 0.65 μm pore). An alternative perfusion apparatus was described by Mulder *et al.*, (1975), in which the preloaded synaptosomes were layered onto a mini Sephadex column.

Numerous adaptations have been made to study the release of different neurotransmitters in response to varying stimuli; such changes have included:

i) the type of support;

ii) the flow rate;

iii) the size of the collected fraction;

iv) the period of stimulation.

3.1.1 The period of stimulation

Perfusion studies have been carried out by several groups in which the period of stimulation has lasted several minutes (e.g. Raiteri *et al.*, 1974; Mulder *et al.*, 1975). Alternative studies have used continual stimulation e.g. the electrical stimulation of slices, Richards (1985) or K^+ depolarisation, Frankhuyzen and Mulder (1982). Other groups have studied events over short (< 30 s) periods, e.g. Redburn *et al.*, (1975), Drapeau and Blaustein (1985), Minnema and Michaelson (1985).

3.1.2 Calculation of release data

There are few reports of methods for calculating the release from repeatedly stimulated synaptosomes. It is more usual to stimulate once and compare the S_1 values obtained in parallel drug-treated and control systems (e.g. Minnema and Michaelson, 1985; Drapeau and Blaustein, 1983).

More studies have been carried out using perfused slice preparations (e.g. Ennis *et al.*, 1981; Gillet *et al.*, 1985; Arbilla and Langer, 1984; Frankhuyzen and Mulder, 1982) where the efflux is usually expressed as a fractional release rate, that is, the radioactivity in each fraction divided by the total amount of radioactivity present in the slice at the start of the collection period. The basal fractional release is then subtracted to give the stimulated release rates. The tissue is usually stimulated twice (e.g. Ennis *et al.*, 1981; Arbilla and Langer, 1984) and the radioactivity released by the two pulses (S_1 and S_2) expressed as the ratio S_2/S_1. To study the effect of drugs on stimulated (K^+ or electrical) release, the drug is introduced for a short period before the second stimulation. The

ratios S_2/S_1 for both the control and drug treated slice are compared. The effect of the drug on the basal efflux can also be determined by comparison of the basal release before S_2 with the basal release before S_1 (Arbilla and Langer, 1984).

One of the original aims of this work was to produce a synaptosomal perfusion system in which repetitive stimulation could be carried out using small pulses of stimuli followed by simple analysis of the released radioactivity.

3.1.3 The original perfusion system

The work described in this thesis was developed from a simple synaptosomal perfusion system described by Mills and Wonnacott (1984). Their conditions were similar to those originally described by Raiteri *et al.* (1974). The perfusion system consisted of a pyrex glass perfusion chamber, modified from McKernan and Campbell (personal communication). The chamber was cylindrical (3 cm diameter) with a circumferential glass ridge to support the filter disc onto which the preloaded synaptosomes were placed (Fig. 3.1). To perfuse medium through the chamber, tubing was attached above and below the filter support.

Modified Krebs-bicarbonate medium, pH 7.4, containing 0.01 mM pargyline and 2 mM ascorbic acid (Mills and Wonnacott, 1984) maintained at 37°C by placing the reservoir in a water bath close to the chamber, was pumped through each chamber using a multichannel peristaltic pump (Gilson Minipuls 2). The inlet flow rate was 1 ml/min and the outlet flow rate was 2.5 ml/min, this difference prevented any build up of medium on or below the filter.

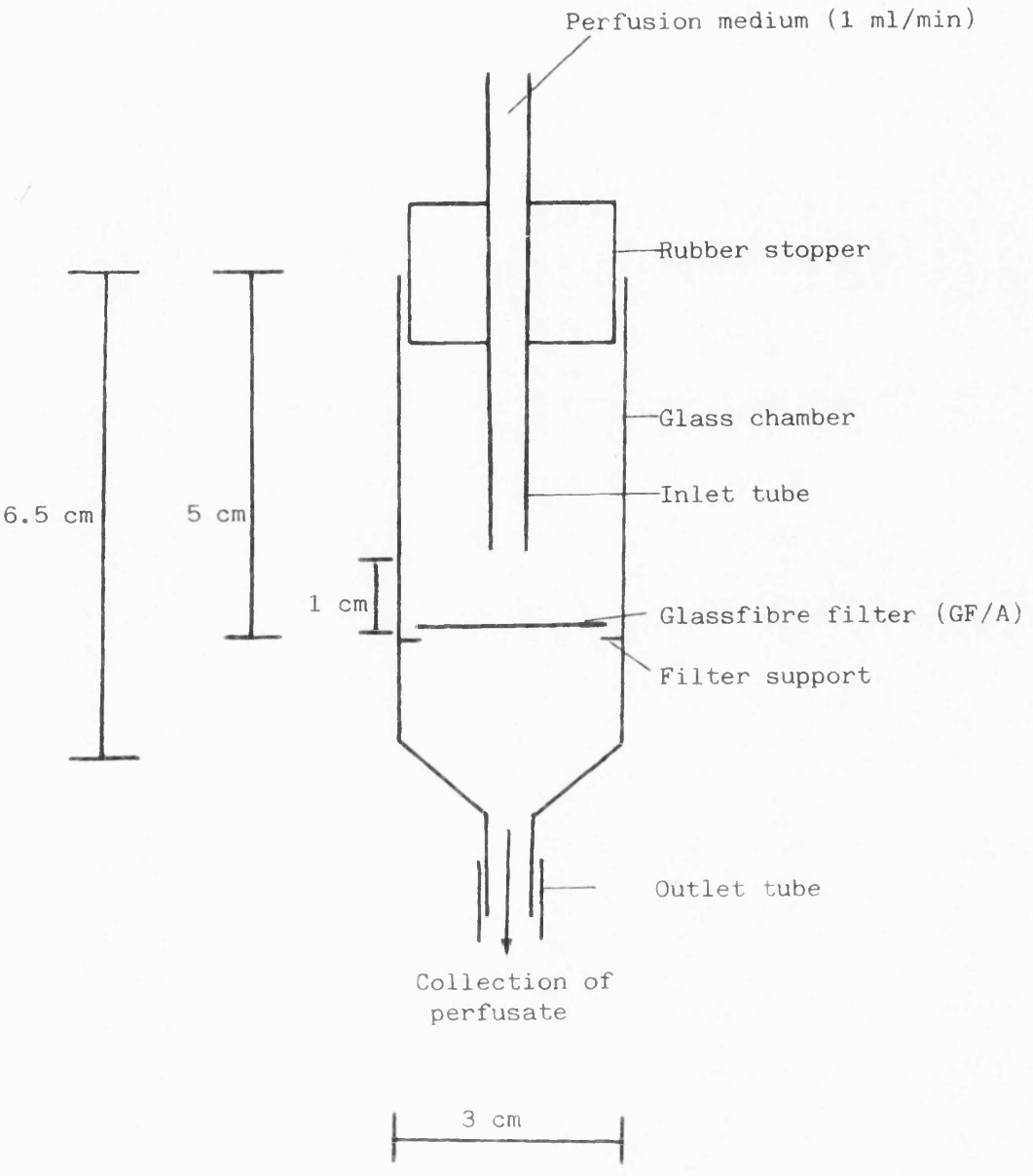


Fig. 3.1. The original perfusion chamber.

The striatal synaptosomes were prepared (Section 2.2.1, p. **63**) and preloaded with radioactive DA by incubation with [2,5,6-^{3}H]DA (specific activity 6.0 Ci/mmol, final concentration 0.2 μM), for 30 min at 37°C. Samples (1 ml, equivalent to 100 mg wet wt. of orginal tissue) were layered onto glass fibre filters (Whatman GF/A, 2.5 cm) by gentle suction and placed in the perfusion chamber. The inlet tubing was positioned 1 cm above the filter and perfusion was carried out for 40 min. After this period the basal (spontaneous) release rate of [^{3}H]DA had reached a constant value.

The effect of drugs on [^{3}H]DA release was tested by introducing a 4 min pulse of test substance into the perfusion medium. To prevent mixing, the pulse was separated from the perfusion medium by air gaps. A minimum interval of 12 min perfusion with normal medium was allowed between each test pulse. Fractions (4 ml) of the perfusate were collected manually and counted for radioactivity in scintillant (Triton X-100/toluene, 1:2 containing 0.3% (w/v) PPO). The radioactivity remaining on the filters at the end of the perfusion experiment was also measured and used to calculate the percentage of the total radioactivity that was released. Two chambers were run in parallel to allow simultaneous perfusion of both treated and control synaptosomes.

Using such a system the release of [^{3}H]DA from striatal synaptosomes could be evoked by nicotine in a dose-dependent manner (Mills and Wonnacott, 1984). The release by nicotine (100 μM) was also shown to be specifically blocked by the ganglionic antagonist mecamylamine (10 μM). However, the concentrations of agonists required to elicit a response in these preliminary experiments were high and it was found that the administration of mecamylamine alone at concentrations

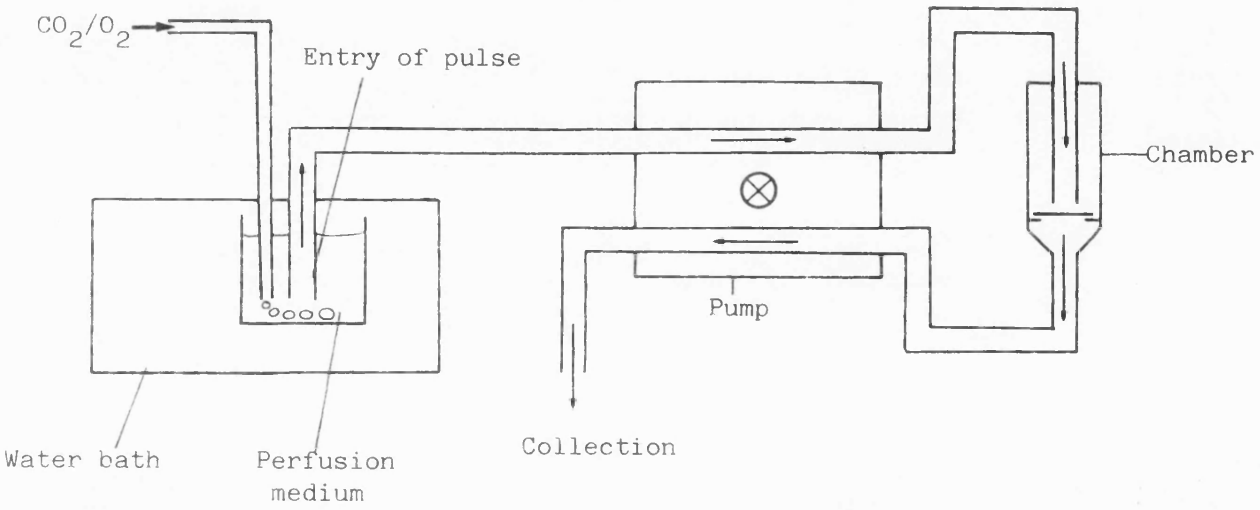


Fig. 3.2. The original perfusion system (not to scale).

greater than 10 μM caused an increase in [^{3}H]DA release. Additionally, practical difficulties were encountered, of which the major one was the manual collection of fractions of perfusate at the same time as introducing a pulse of drug.

The following section describes the successive changes that were made to this original system with the aim of optimising and improving its sensitivity and reproducibility.

3.2 DEVELOPMENT OF THE PERFUSION SYSTEM

3.2.1 Modifications (i)

Striatal synaptosomes preloaded with [^{3}H]DA of high specific activity using the optimum conditions defined in Section 2.3.5, were initially perfused using the original system of Mills and Wonnacott (1984). The only changes to the experimental procedure were the substitution of the original type of glass fibre filter (Whatman GF/A) for a GF/B grade and a reduction in the amount of protein loaded onto each filter (0.7 ml synaptosomes instead of 1 ml). The GF/B filter has a smaller particle retention size and is thicker than the GF/A grade (Table 3.3). The advantage of this is the retention of a greater number of synaptosomes, the average size of a synaptosome being 0.5 - 2 μm (Jones, 1975; Whittaker, 1984; see also Section 2.3.1).

An early perfusion profile is shown in Fig. 3.3. The synaptosomes were washed with perfusion medium (modified Krebs bicarbonate medium, pH 7.4 containing 0.01 mM pargyline and 1 mM ascorbic acid, p.**135**). After a 40 min washout period the synaptosomes were challenged with two 4 ml pulses of 100 μM-nicotine, designated S_1 and S_2; an interval of 16 min separated the pulses. In a parallel perfusion system the synaptosomes were continually washed with normal perfusion medium without exposure to nicotine. This release was taken as the basal release and was subtracted from that obtained in the system challenged with nicotine. Throughout the development of the perfusion system the naturally occurring nicotine isomer, (-)-nicotine was used to evoke transmitter release. The preliminary experiment shown in Fig. 3.3 clearly showed that nicotine at high concentrations evoked the release of [^{3}H]DA from preloaded striatal synaptosomes. The elevated radioactivity in response to stimulation was expressed as the total

dpm above spontaneous efflux, which was then converted to fmoles [^{3}H] DA released per mg synaptosomal protein by reference to the specific activity of the radiolabelled dopamine (10^5 dpm/pmole) and the protein concentration of the synaptosome preparation. Alternatively, the peak of radioactivity was calculated as the percentage of the total tritium present on the filter immediately prior to stimulation. Because the monoamine oxidase (MAO) inhibitor, pargyline, and the antioxidant ascorbic acid were present in the perfusion medium, little degradation of the released [^{3}H]DA was expected. The tritium present in the perfusate was therefore taken as representing [^{3}H]DA. This was confirmed in later studies (Section 3.3.3).

The initial perfusion experiments demonstrated that nicotine (100 μM) could repeatedly evoke the release of [^{3}H]DA from striatal synaptosomes. However, apart from the practical difficulties of the system (p.**115**), the sensitivity had not been greatly improved as high concentrations of nicotine (100 μM) were required to elicit [^{3}H]DA release. In an attempt to enhance the nicotinic effect the following changes were made.

In order to reduce the washout period, during which time the synaptosomes may begin to deteriorate, the preloaded synaptosomes were washed by resuspension in 2 volumes of perfusion medium followed by centrifugation in a bench centrifuge at 1,000 *g* for 7 min. The resulting pellet was gently resuspended in medium (using a Pasteur pipette) to its original concentration. The integrity of the synaptosomes was maintained using this method (p.**100**). The washed synaptosomes were layered onto the filters (as in the previous procedure) but the flow rate was reduced to 0.5 ml/min, the size of the drug pulse to 1 ml, and smaller fractions were collected. Providing

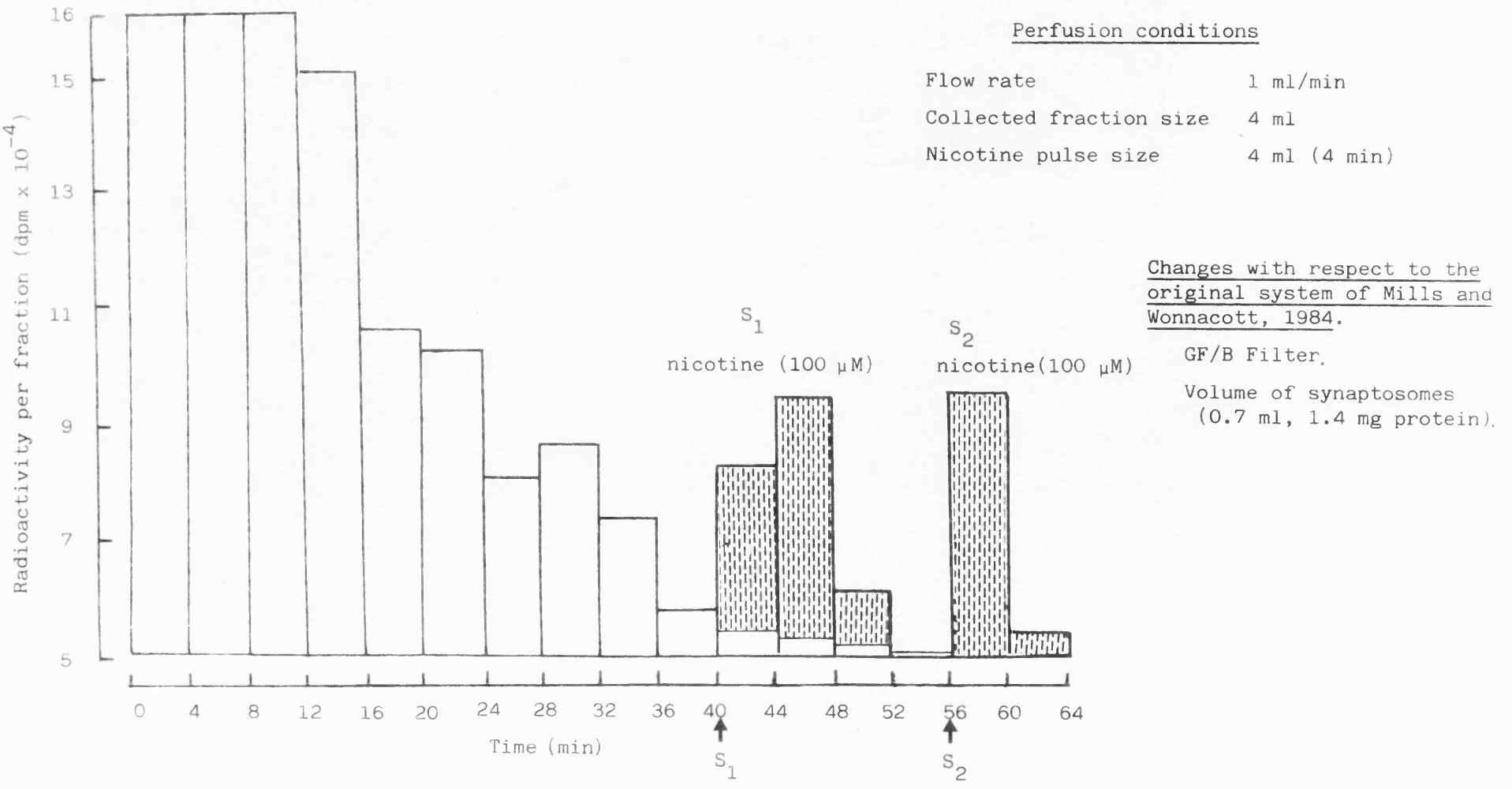


Fig. 3.3. Perfusion profile I, showing radioactivity released per 4 min fraction. Shaded area represents release above the spontaneous (unshaded) level.

Pulse	Total amount of nicotine (nmoles)	fmoles [^{3}H]DA released above basal/mg protein	fmoles [^{3}H]DA/mg protein/ nmole nicotine	Evoked release (above basal) as percentage of radioactivity remaining on filter before stimulation
S_1	400	556	1.4	4.6
S_2	400	404	1.0	4.0

Table 3.1. Release of [^{3}H]DA by nicotine using the perfusion conditions described above in Fig. 3.3.

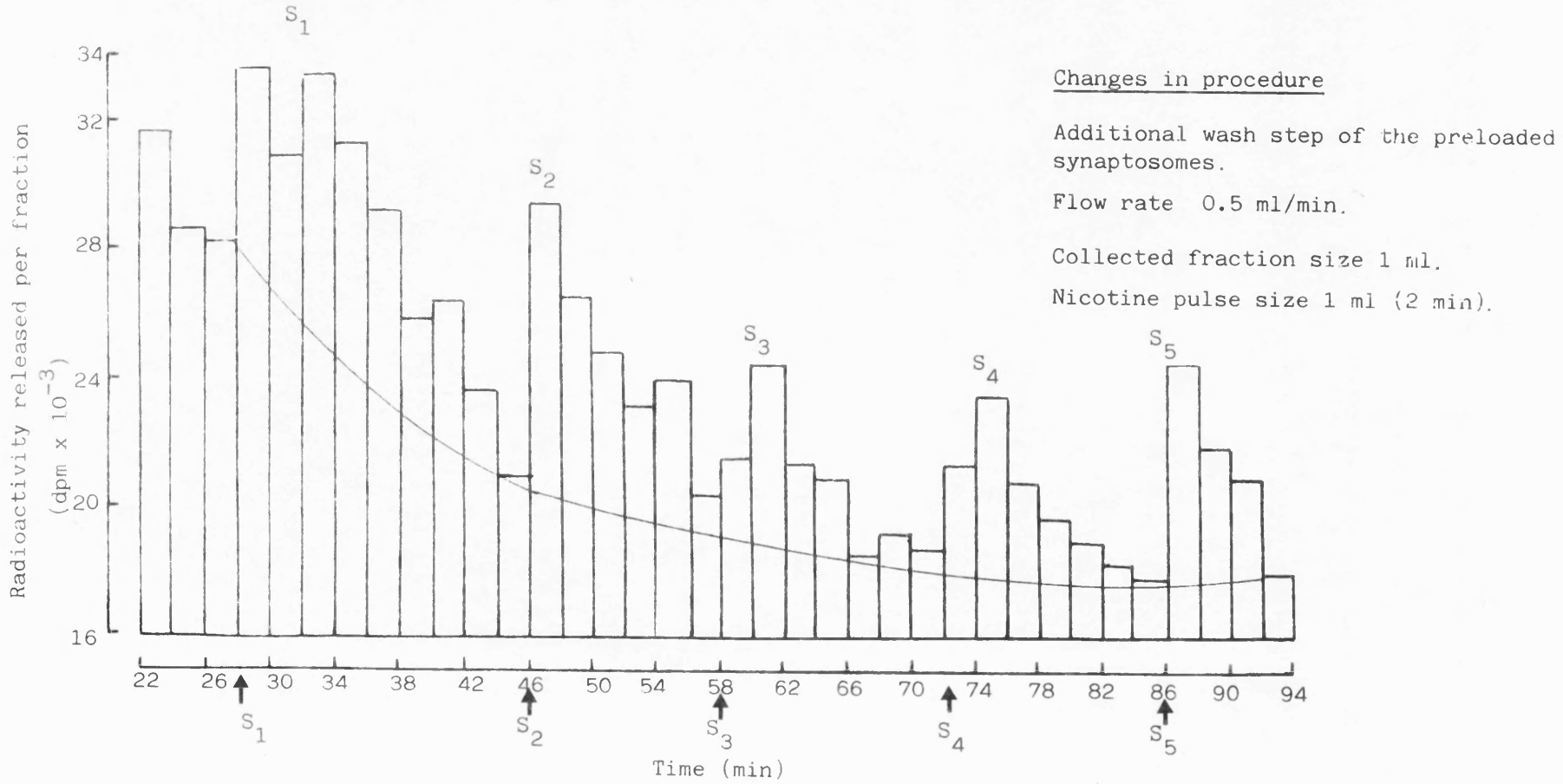


Fig. 3.4 Perfusion profile II.

Pulse	Total amount of nicotine (nmoles)	fmoles [^{3}H]DA released above basal/mg protein	fmoles [^{3}H]DA/mg protein /nmol nicotine	Evoked release (above basal) as percentage of radioactivity remaining on filter before stimulation
S_1	100	306	3.1	2.9
S_2	"	201	2.0	3.0
S_3	"	114	1.1	2.0
S_4	"	137	1.4	2.9
S_5	"	126	1.3	3.5

Table 3.2. Release of [^{3}H]DA by nicotine using the perfusion conditions described in Fig. 3.4.

Data presented are from a single experiment typical of six similar experiments.

that the additional wash step was included, a 40 min washout period was still sufficient despite the slower flow rate.

The combined effect of these changes on transmitter release is shown in Fig. 3.4. Nicotine (1 ml, 100 μM) clearly enhanced the efflux of radioactivity and the sensitivity of the revised system (as indicated by fmoles [^{3}H]DA released/mg protein/nmole nicotine; see p. **138**) had doubled. However, the concentration of agonist required to observe a convincing peak above the basal level was still very high (100 μM). The possibility that the poor resolution of the peaks was a consequence of mixing of the released radioactivity in the tubing was investigated by injection of a 10 μl sample of [^{3}H]DA (0.1 μCi) below the filter followed by normal washing. Fractions (1 ml) were collected and the radioactivity measured. 97.5% of the total counts were recovered in one fraction, indicating that no significant mixing occurred.

The system described above gave satisfactory qualitative evidence of nicotine-induced dopamine release. However, further modifications were made in an attempt to improve the sensitivity and resolution.

3.2.2 Comparison of filters

Two types of filters have been used to support synaptosomes in perfusion chambers, i) glass fibre (Levy *et al.*, 1973; Collard *et al.*, 1981, and Mills and Wonnacott, 1984), and ii) cellulose acetate/nitrate (Raiteri *et al.*, 1974; Minnema and Michaelson, 1985). The glass fibre filters have a high loading capacity and rapid flow rate compared with cellulose filters of a similar particle retention size. However, the latter type of filter has been shown to be more

Grade		Retention size (µm)	Thickness (mm)	Radioactivity remaining after washing sample % total dpm	Void volume (ml)
Whatman Glassfibre	GF/A	1.6	0.26	0.81 ± 0.17	ND
	GF/B	1.0	0.68	0.39 ± 0.09	ND
	GF/F	0.7	0.42	0.41 ± 0.12	0.16 ± 0.02
	GF/D	0.65	0.68	ND	0.18 ± 0.02

Table 3.3. Comparison of filters.

Binding of [^{3}H]DA to the filters was determined by placing a 10 µl sample of [^{3}H]DA (0.1 µCi) on each type of filter positioned in a Millipore single filtration unit and washing with 6 ml perfusion medium. The radioactivity remaining on the filters was counted (p.**74**) and expressed as a percentage of the total radioactivity. Estimation of the void volume was carried out by weighing each filter dry and then after soaking in water. The wet filters were placed in Eppendorf tubes and centrifuged at high speed in a bench centrifuge for 5 min. The excess water was removed and the filters reweighed. The difference in the final weight and the dry weight was taken as the void volume. Results are expressed as the mean ± SEM (n=4).

efficient in DA uptake studies using vacuum filtration, (Schoemaker and Nickolson, 1983).

For the perfusion of synaptosomes a filter support was required with a high loading capacity, low specific binding of radiolabelled transmitters and a rapid flow rate. The properties of a range of glass fibre filters were compared with those of a Millipore cellulose filter (0.65 µm). The results are summarised in Table 3.3.

3.2.3 Modifications (ii)

A Millipore single unit pyrex filtration apparatus (from Millipore, cat. no. OXX10 22500), was investigated as a new model for the perfusion system. The lower part consisted of a scintered glass filter support, the bottom of which had been drawn out so the tubing could be attached and medium pumped through the chamber as in the previous arrangement.

The upper and lower parts of the chamber were held together by an aluminium spring clamp. A filter soaked in perfusion medium was firmly supported between the two halves. Two types of filter were compared, i) a cellulose acetate/nitrate (Millipore, 0.65 µm) and ii) a glass fibre filter (Whatman GF/F, 0.7 µm), see Table 3.3.

However, the former type of filter proved difficult to work with as high pressures were required to ensure rapid flow through the filter. The binding of [^{3}H]DA to the GF/F filter was comparable with that of the GF/B grade; because the GF/F filter had a smaller retention size than the GF/B it was chosen for use in all subsequent perfusions.

Distribution of a 1 ml drug sample through the revised system was determined by the perfusion of a pulse of dye (Blue dextran,

Pharmacia). The sample was collected in a total of 4 (1 ml) fractions. However, examination of nicotine-evoked DA release showed that although the system gave consistent results, the sensitivity had not been improved.

To study the release in greater detail using the slower flow rate of 0.5 ml/min, a smaller volume of synaptosomes (0.4 ml) was loaded onto the filter (to limit the possibility of reuptake of the released transmitter, Collard *et al.*, 1981) and the size of pulse was reduced to 100 µl. This pulse was introduced into the system via a 'T' junction tap fixed into the tubing just before entry into the chamber (Fig. 3.5). Administration of the drug by this method was quick and the response was greatly enhanced (Fig. 3.6).

The next aim was to investigate the effect of reducing the collection volume on resolution. Fraction collectors (LKB 2112 Redirac) were used to automatically collect perfusate (2-8 drops) in plastic minivials (volume 6 ml) placed in the cassette of the fraction collector. The radioactivity in each vial was counted (p.**74**) using a scintillant of high aqueous capacity.

To allow assembly of the chambers directly above the arm of the fraction collectors the method of the removal of perfusate was changed. Instead of suction through tubing below the filter, air pressure was applied above the filter (Figs. 3.7, 3.8 and 3.9). However, very high air pressure was required to ensure adequate flow through the filter and this also disturbed the flow of the incoming medium. To overcome these problems the scinter was removed from the lower part leaving a rim to support the filters and a smaller volume (250 µl) of synaptosomes was used. To protect the synaptosomes from the impact of the drops of medium, a prefilter (Whatman GF/D, cut to

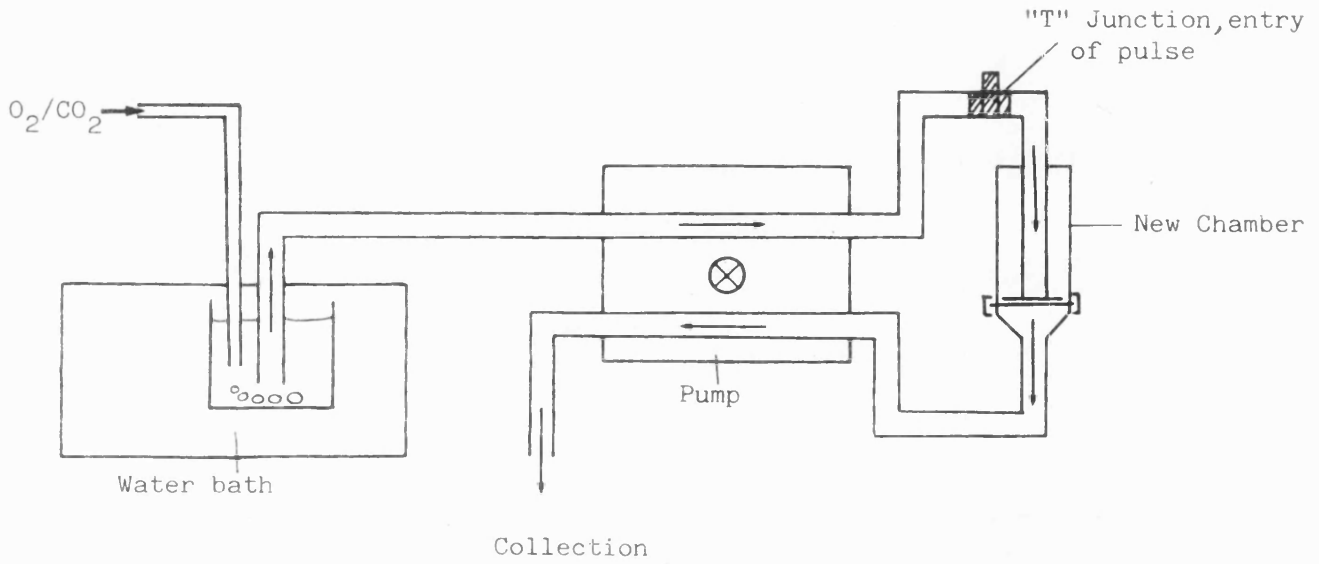


Fig. 3.5. Modified Perfusion Apparatus.

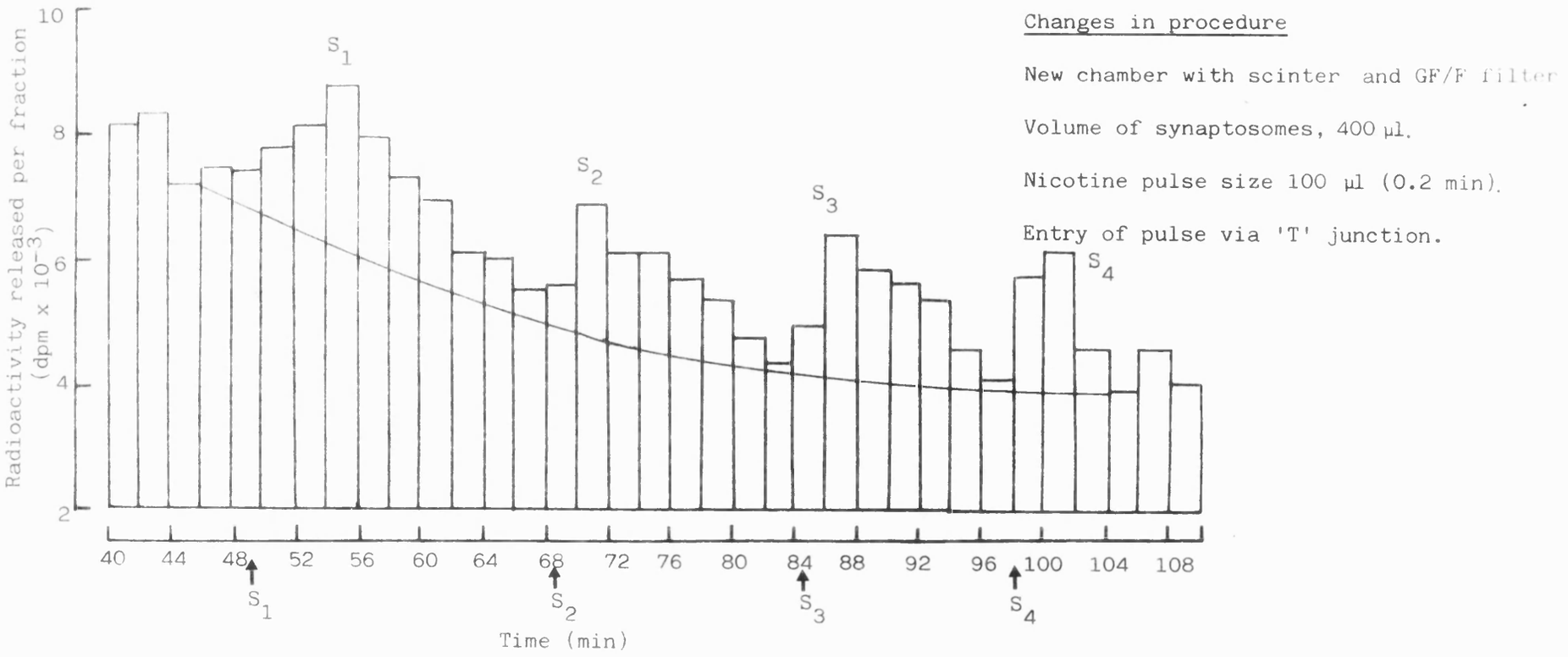


Fig. 3.6. Perfusion Profile III.

Pulse	Total amount of nicotine (nmoles)	fmoles [^{3}H]DA released above basal/mg protein	fmoles [^{3}H]DA/mg protein /nmole nicotine	Evoked release (above basal) as percentage of radio-activity remaining on filter before stimulation
S_1	10	156	15.6	2.2
S_2	"	105	10.6	1.7
S_3	"	111	11.1	1.2
S_4	"	74	7.4	1.4

Table 3.4. Release of [^{3}H]DA by nicotine using the perfusion conditions described in Fig. 3.6.

Results shown are representative of 3 experiments.

1.5 cm) of high retention size and flow rate was placed over the bed of synaptosomes. The tube containing the incoming perfusion medium was brought into contact with the centre of the filter. In view of the improved resolution from reducing the flow rate of the medium (Fig. 3.4), the flow rate was further reduced to 0.175 µl/min.

With these modifications the system was more sensitive. Nicotine (50 µM) evoked release of the radiolabelled transmitter and this was clearly observed above the basal release (Fig. 3.10). In the experiment shown, small fractions (4 drops) were collected with the result that the peaks spread over only 3 to 4 fractions. However, to reduce the total number of samples to be counted in subsequent experiments larger fractions (8 drops) were collected resulting in peaks being spread over 5 to 6 fractions. In the experiment shown in Fig. 3.10 the washout period had been reduced to only 20 min and clearly the basal release had not settled down during this time period. In all subsequent experiments the wash out period was 40 min. The overall sensitivity of the system had been greatly improved, the response to nicotine having increased by a factor of 80 (see p.**138**).

Unfortunately the introduction of a drug via the 'T' junction required careful manipulation, and mixing often occurred leading to the dilution of the sample. Direct injection of the sample into the flow of the perfusion medium was also carried out but this lead to mixing and leaking at the point of injection. The administration of a pharmacological agent was therefore introduced as a pulse (as in the original experiments p.**111**). For a 100 µl pulse (flow rate 150 µl/min) a 40 sec pulse of drug was separated by 30 sec air gaps. This method ensured that there was no dilution of the drug sample. A change in perfusion medium was also achieved by the transfer of tubing from

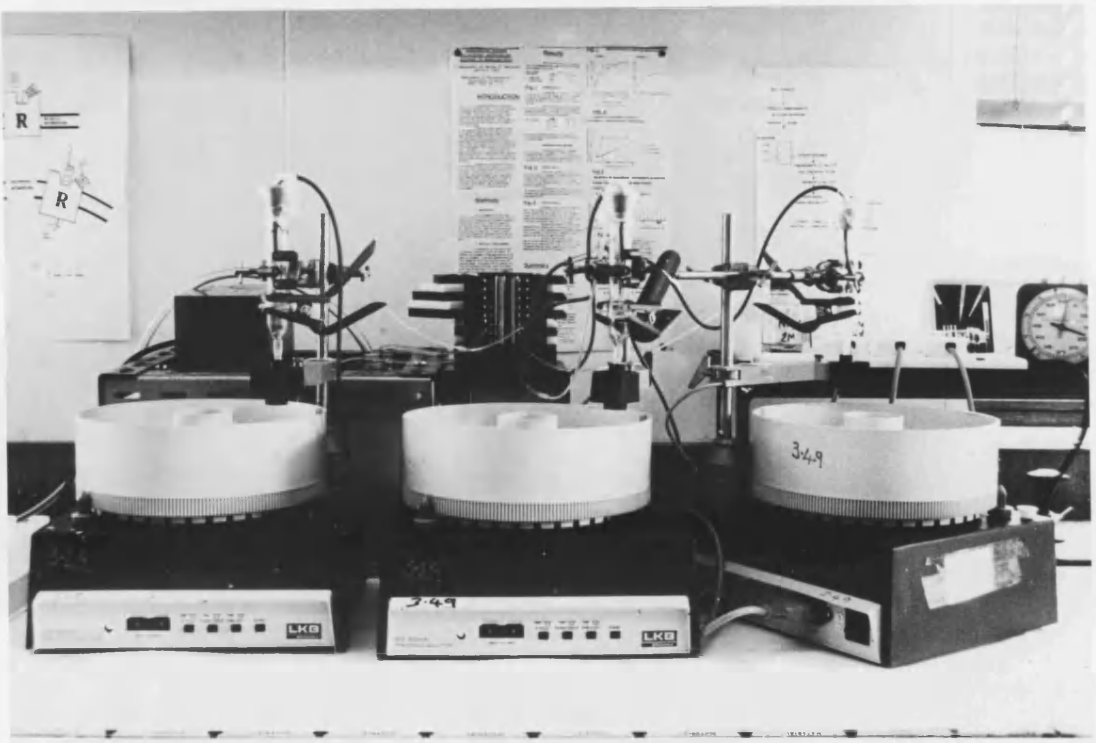


FIG.3.7 The final perfusion apparatus

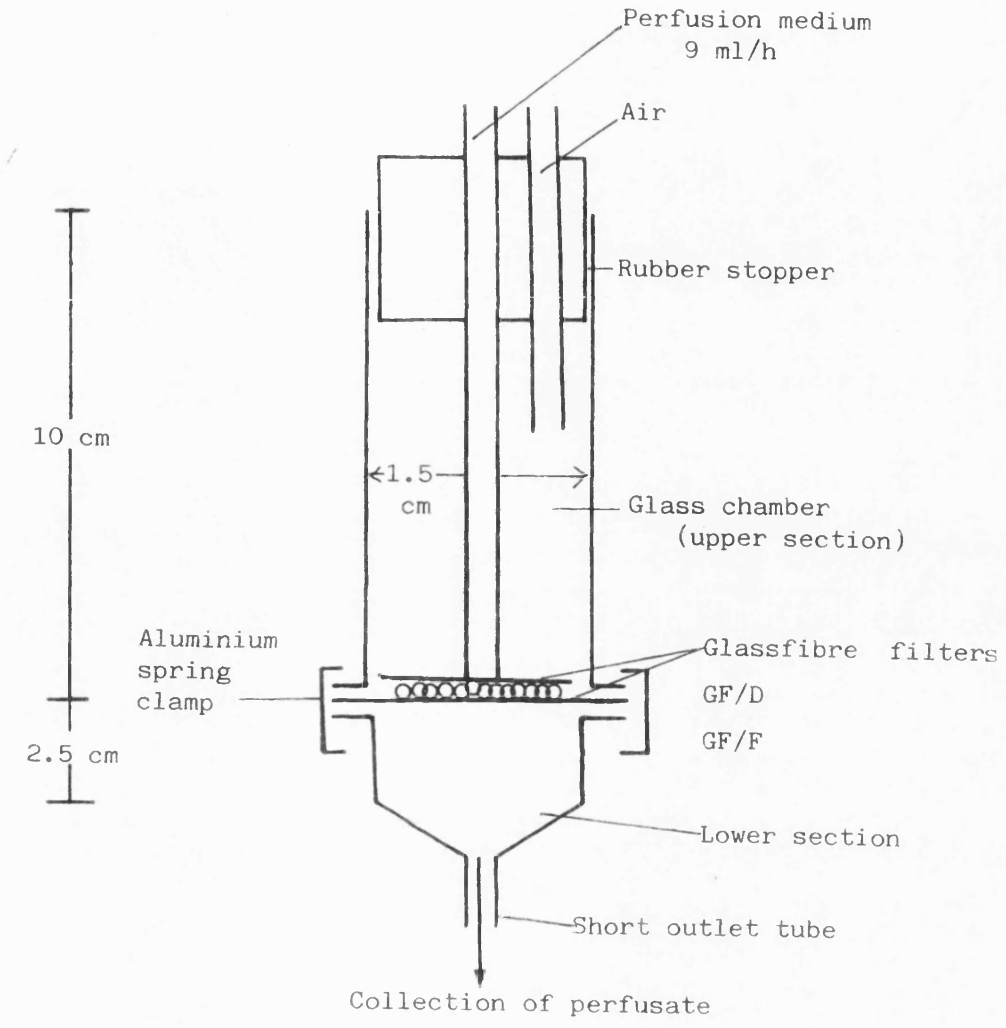


Fig. 3.8. The final perfusion chamber.

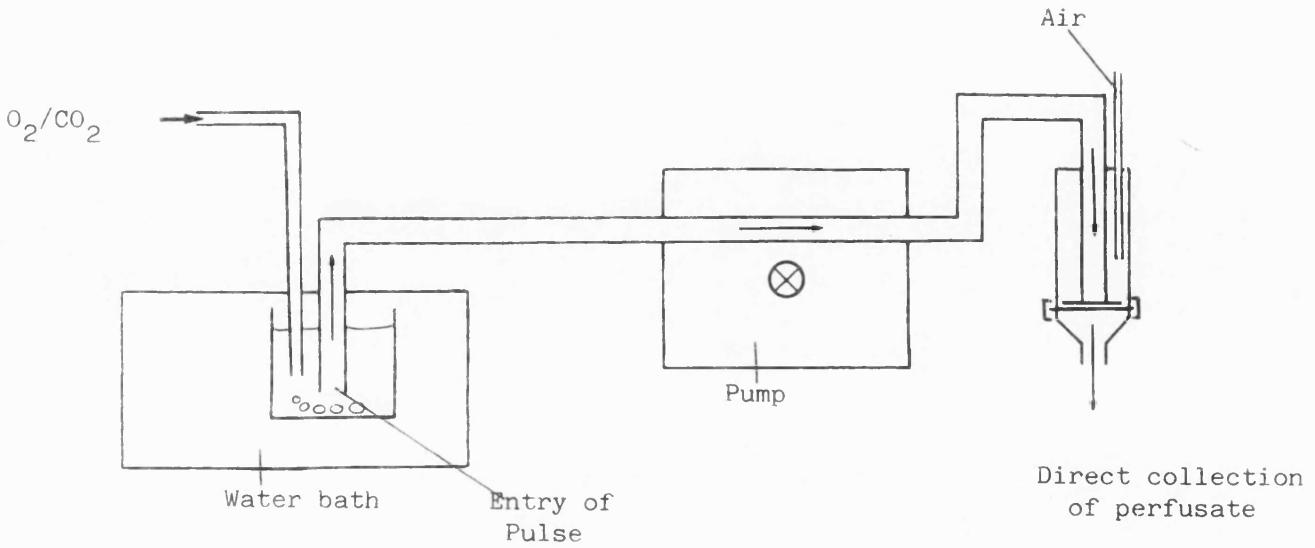
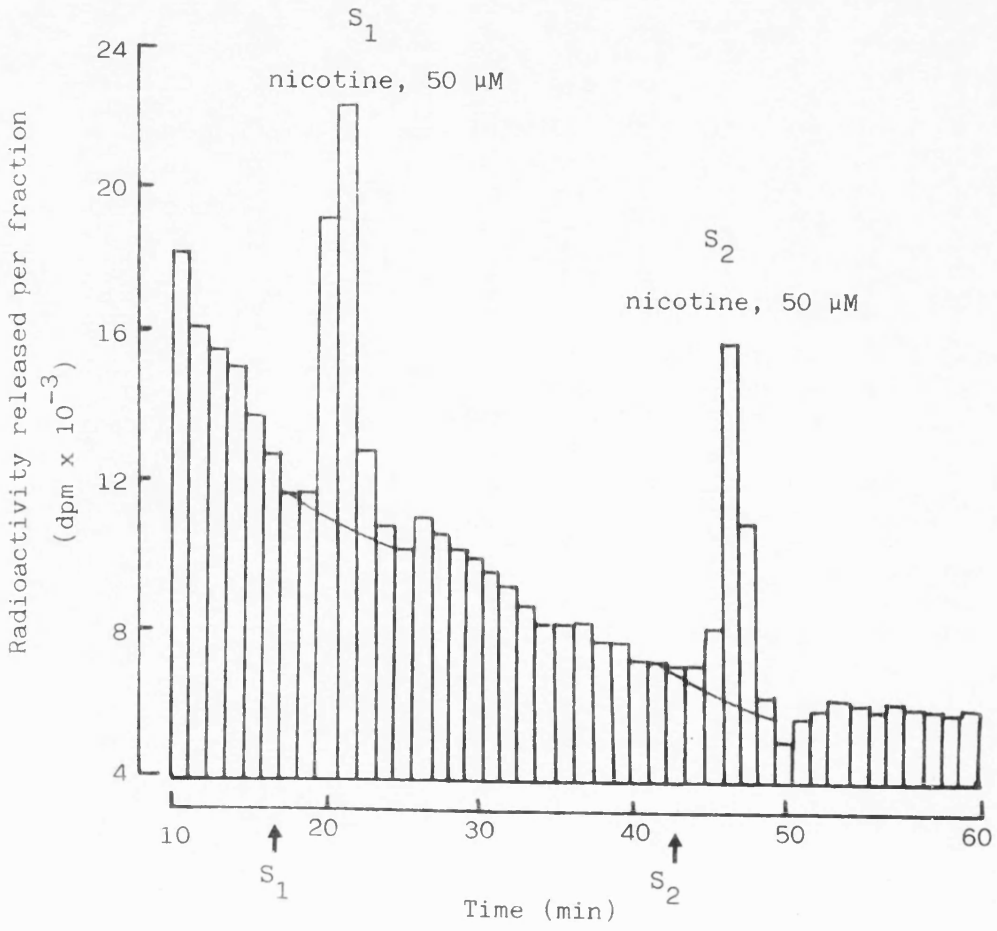


Fig. 3.9. The final perfusion arrangement.



Changes to perfusion procedures

Absence of scinter in chamber.

Additional prefilter (GF/D).

Air pressure in upper chamber.

Automatic collection of fractions (4 drops

Volume of synaptosomes, 250 µl.

Flow rate 175 µl/min.

Fig. 3.10. Perfusion Profile IV.

Pulse	Total amount of nicotine (nmoles)	fmoles [^{3}H]DA released above basal/mg protein	fmoles [^{3}H]DA/mg protein /nmole nicotine	Evoked release (above basal) as percentage of radio-activity remaining on filter before stimulation
S_1	5	408	81.6	2
S_2	5	311	62.2	2

Table 3.5. Release of [^{3}H]DA by nicotine (50 μM) in Fig. 3.10.

Results shown are representative of 3 experiments.

one bottle to the next in the water bath, an air gap separating the two media. The introduction of air gaps caused no artifactual changes in [^{3}H] DA release.

3.2.4 The final system

a) The perfusion apparatus

The final perfusion apparatus was composed of a multichannel peristaltic pump to supply medium to 4 perfusion chambers, each supported over a fraction collector (see Fig. 3.7 and Table 3.6).

b) Perfusion Medium

The perfusion medium was a modified Krebs-Ringer-bicarbonate solution, composition shown below.

Substance	Final concentration (mM)
NaCl	118.5
KCl	2.4
$CaCl_2.2H_2O$	2.5
KH_2PO_4	1.2
$MgSO_4.7H_2O$	2.5
$NaHCO_3$	24.9
Glucose	10
Ascorbic acid	1
Pargyline	0.01

Stock solutions (x10 final concentration) were prepared and stored at 4°C for a maximum of one month. Glucose, ascorbic acid and pargyline solutions were freshly prepared. Equal volumes of the stock solutions

Table 3.6. Details of the perfusion apparatus.

Component	Name/dimensions	Supplier
Peristaltic pump	Gilson Minipuls 2	Anachem, Luton, Beds., U.K.
Peristaltic pump tubing	I.D. 2.00 mm	"
Translucent vinyl tubing	I.D. 2.00 mm O.D. 3.00 mm	Portex 62600, Berck-sur-Mer, France.
Fraction Collector	LKB 2112 Redirac	LKB Instruments, South Croydon, Surrey, U.K.
Chamber	Glass (12.5 cm)	University workshop (also available from Millipore Limited.)
Aluminium spring clamp	-	Millipore Ltd. Harrow, Middx. U.K.
Filters	GF/D 2.5 cm GF/F 2.5 cm	Whatman Biochemicals Ltd., Maidstone, Kent, U.K.

were mixed and distilled water added to give the required final concentrations. The medium was continually gassed with O_2/CO_2 (95%/5%). After 1h gassing the pH was adjusted to 7.4 with 0.1M NaOH.

The MAO inhibitor pargyline and the antioxidant ascorbic acid were included to reduce degradation of DA. The KCl concentration used was half that originally used by Mills and Wonnacott (1984). This was to reduce the total concentration of the depolarising K^+ ion and to achieve a K^+ concentration which is near the optimum of 3.9 mM for DA uptake (Holz and Coyle, 1974). Ca^{2+}-free medium was prepared by omitting $CaCl_2$, osmolarity being maintained by substitution with NaCl. Reduced Na^+ medium was obtained by the replacement of NaCl with sucrose (237 mM).

When solutions containing elevated concentrations of KCl (release studies) were used a corresponding reduction in the NaCl concentration was made to maintain osmolarity.

c) The final perfusion conditions (V)

Synaptosomes (100-250 µl) preloaded with [^{3}H] DA were layered onto Whatman GF/F filters, wetted in perfusion medium, positioned in the perfusion chambers. A prefilter (GF/D) was placed over the synaptosomes and perfusion medium (37°C) was pumped over the filters at a flow rate of 150 µl/min. Air pressure (20 p.s.i.) was applied above the filters to ensure rapid removal of the perfusate. After a 40 min washout period, the synaptosomes were exposed to pulses (100 µl) of pharmacological agents, the pulse being separated from the main flow of medium by air bubbles. Fractions (8 drops, 340 µl) of the perfusate were collected 10 min prior to the first stimulation and thereafter until the end of the experiment. The radioactivity in each

fraction and filter was counted (p.**74**) and a perfusion profile constructed.

At the end of each experiment the tubing and chambers were thoroughly washed with distilled water. Every 4 weeks the tubing was replaced and the flow rate calibrated.

The details described above constituted the final system used throughout the rest of the study.

3.2.5 Discussion of the final system

a) Improved sensitivity

The responsiveness of each perfusion system during its development, expressed as fmoles [^{3}H] DA released (above spontaneous release) /mg protein/nmole nicotine, can be used as an index of the sensitivity of the system. Thus the sensitivity of the final system was clearly greatly increased (Table 3.7), with almost a 100 fold increase in the amount of [^{3}H] DA released per nmole nicotine.

The concentration of nicotine (100 µM) used throughout the development of the perfusion system was high. In later studies the effect of nicotine over the range 0.01 µM-1 mM was studied (p.**185**) and concentrations above 100 µM were found to elicit maximum [^{3}H] DA release. In perfusion system IV, the concentration of nicotine used was non-saturating (50 µM) and therefore the index of sensitivity is not directly comparable with the other systems.

b) Practical changes

The small volume of synaptosomes loaded onto each filter (150 µl, approx. 0.4 mg protein) allowed the number of chambers in operation to be increased from 2 to 4 without increasing the number of

System	Nicotine conc^{n}	Flow rate ml/min	Length of nicotine pulse (min)	Release fmoles [^{3}H]DA/mg protein/nmole nicotine	Release fmoles/mg/nmole /min
I. Fig. 3.3	100 µM	1.0	4.0	1.4	0.35
II. Fig. 3.4	100 µM	0.5	2.0	3.1	1.55
III. Fig. 3.6	100 µM	0.5	0.2	15.6	78
IV. Fig. 3.10	50 µM	0.175	0.57	81.6	143
V. Final system	100 µM	0.15	0.66	69	105

Table 3.7. Development of a perfusion system.

rats required; two rats were used for a striatal preparation and one rat was used for a hippocampal preparation (Section 5.2.1). Because of the limited availability of scintillation counters it was necessary to reduce the total number of samples for radioactivity counting. Therefore 8 drop fractions (340 µl) of perfusate were collected resulting in a slight reduction in the resolution of the peaks of released radioactivity.

c) The period of stimulation and the time interval between successive stimulations

The length of the period of stimulation (pulse) used in the final perfusion system (p.135) had been reduced from 4 min (4 ml) as in the original system (p.111) to 40 s (100 µl). Using this size of pulse a 20 min recovery time between successive pulses was allowed.

However, although this was an adequate recovery time using nicotine (100 µM) as the stimulus, when the depolarizing agent veratridine (10 µM) was used to evoke the release of DA a much longer interval was required (see Fig. 3.11). K^+ (28 mM) evoked release was also demonstrated (Fig. 3.11). Comparison of the peaks of released radioactivity elicited by the different types of stimuli showed that the response to, and the recovery from nicotine (1 µM) and K^+ (28 mM) were relatively fast compared with the action of veratridine (10 µM).

The differences between the duration of the responses evoked by nicotinic agonists, K^+ and veratridine, and the effect of concentration is dealt with in more detail in Section 4.4.3.

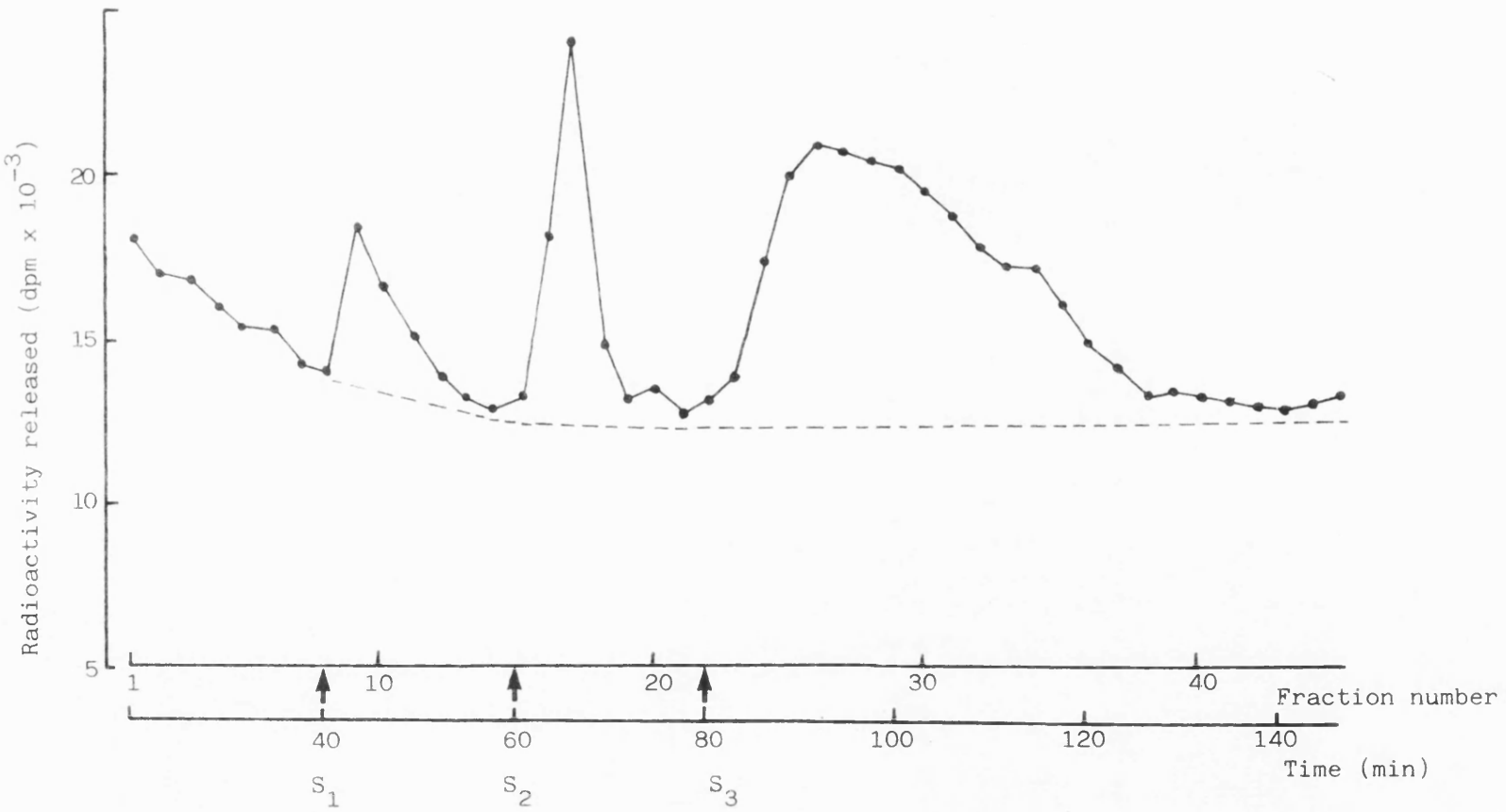


Fig. 3.11. Perfusion profile showing the release of [^{3}H]DA from striatal synaptosomes (200 µl, 0.62 mg protein/filter) in response to S_1 (nicotine, 1 µM), S_2 (K^+, 28 mM) and S_3 (veratridine, 10 µM). ↑ indicates time of arrival of the pulse. Dashed line shows the level of basal release.

3.2.6. Calculation of the release data

a) The effect of repetitive stimulation

Various methods for calculating release data have been reported (see Section 3.1.2).

A method was required for quantifying the [^{3}H]DA released in experiments using the final perfusion conditions which allowed for possible differences between perfusion chambers and day to day variables (e.g. basal release rates).

Throughout the development of the perfusion system the efflux of tritium in response to stimulation was converted to fmoles [^{3}H]DA released per mg protein. Alternatively, the fractional release was calculated.

To compare these two methods, striatal synaptosomes were repeatedly stimulated with increasing concentrations of K^+ (Figs. 3.12 and 3.13). The results show that with time the amount of radioactivity released represents a higher proportion of the amount of radioactivity remaining on the filter (Fig. 3.13b). This is a result of the gradual depletion of the transmitter pool within the synaptosomes with increased length of perfusion time. Maximum release was observed at about 45 mM K^+ and a half maximum release at 29 mM. These results are in agreement with those reported by Minnema and Michaelson (1985) who obtained a half maximum response at 28.5 mM K^+ and a maximum response at 42.75 mM.

In subsequent experiments to measure the effects of increasing concentrations of drug (agonist, K^+ or veratridine), only the release data obtained by the initial stimulation (S_1, t = 40) were used. In experiments in which the synaptosomes were repeatedly exposed to

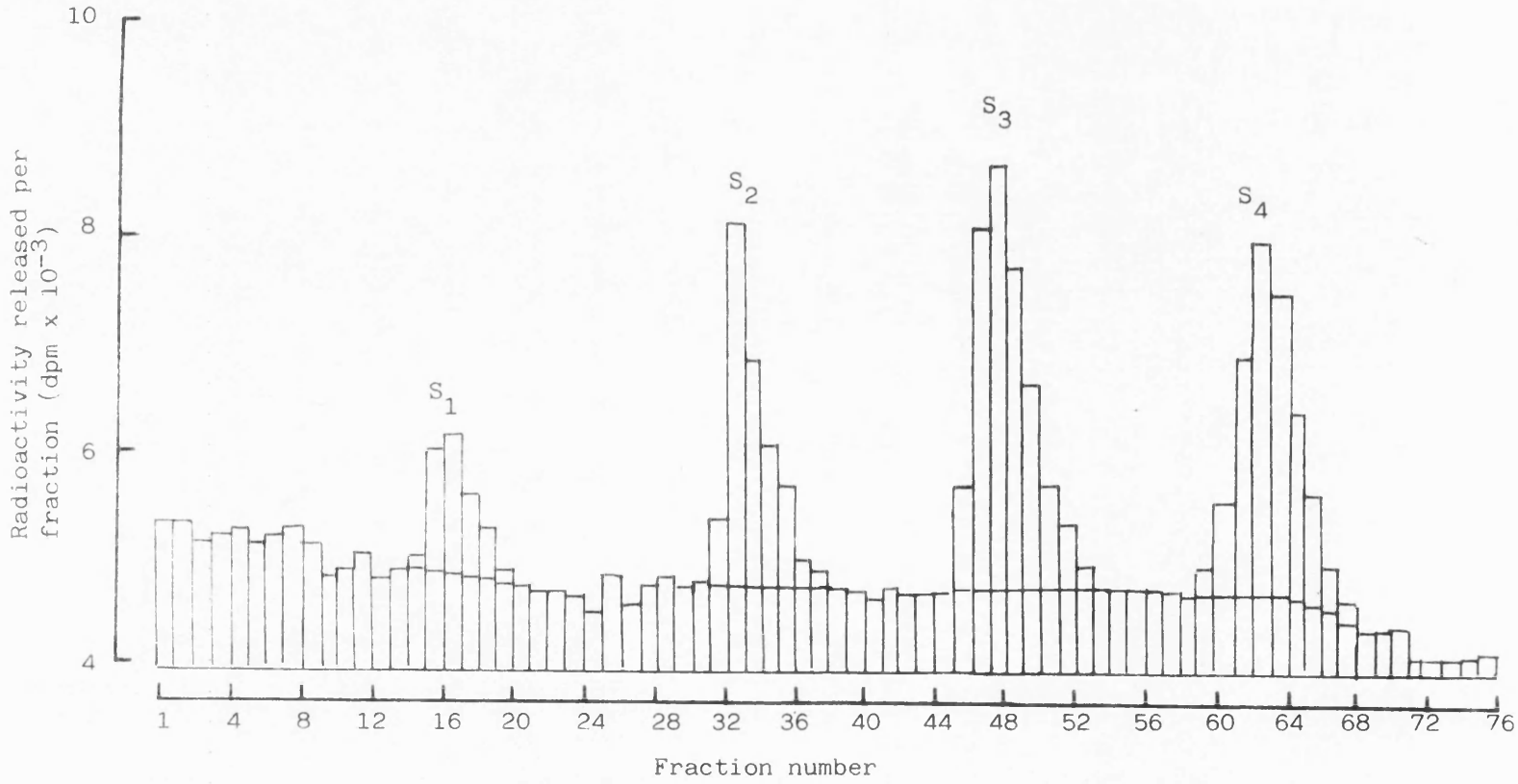


Fig. 3.12. Effect of increasing concentrations of K^+ on [^{3}H]DA release using perfusion conditions V (250 µl synaptosome on filter and 4 drop fractions). After a 40 min washout period, preloaded synaptosomes were stimulated with increasing concentrations of K^+, at 20 min intervals.

Summary of the K^+-evoked release of [^{3}H]DA shown in Fig. 3.12.

Pulse	Concentration of K^+ (mM)	fmoles [^{3}H]DA released/mg protein	Evoked release as a percentage of the radioactivity on the filter (fractional release)
S_1	16	170	0.78
S_2	28	411	2.23
S_3	41	674	4.34
S_4	53	560	4.61

Fig. 3.13a

Dose-response curve
Half maximal = 25 mM

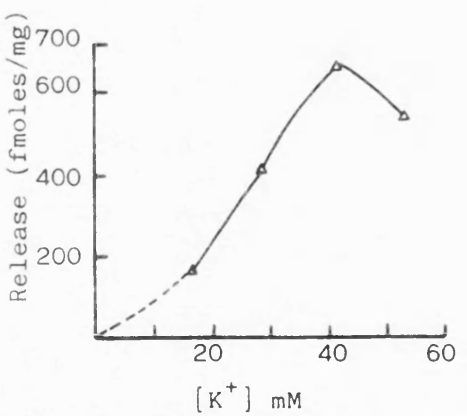
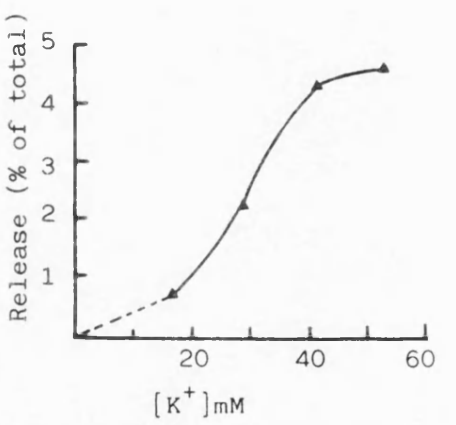


Fig. 3.13b

Dose-response curve
Half maximal = 29 mM



pulses of drugs, stimulation was usually carried out only three times at 30 min intervals after the 40 min washout period.

b) The effect of antagonists on stimulated release: the final perfusion schedule

In early experiments, the effect of an antagonist was studied by running three perfusion chambers in parallel, one chamber acting as a control which was perfused with normal medium, one system to determine basal release which was perfused with normal medium, but which was not stimulated, and one test system in which the perfusion medium contained the antagonist. Towards the end of each experiment a pulse of K^+(28 mM) was administered to standardise each system. The stimulated release in the presence of the antagonist was compared to the control system. However, because there are minor differences in the local conditions within each system, slight differences in release rates between systems were observed.

To overcome this problem the perfusion schedule was changed. Four perfusion systems were operated in parallel and all were initially perfused with normal perfusion medium. As described above, after the 40 min washout period the synaptosomes were stimulated (S_1, t = 40 min) and the stimulation was repeated at t = 70 min and 110 min designated S_2 and S_3 respectively, see Fig. 3.14.

The effect of an antagonist on evoked release was determined by changing the medium in two of the systems to medium containing the antagonist 10 min before S_2 and continuing throughout the S_2 response until 10 min before S_3. The [^{3}H] DA released by the S_2 and the S_3 pulses were expressed as a percentage of the initial stimulation (S_1) to give S_2/S_1 and S_3/S_1, values respectively.

Fig. 3.14. The final perfusion schedule.

A typical perfusion profile showing the time schedule used to study the effect of an antagonist on agonist evoked release. Synaptosomes were stimulated 3 times (S_1, S_2, S_3) at 30 min intervals. The antagonist was introduced into the medium 10 min before and continued until 20 min after S_2. The S_2/S_1 ratio in the presence of an antagonist was calculated as a percentage of the S_2/S_1 ratio obtained in normal medium. Comparison of the S_3/S_1 values gave information about the recovery of the response after exposure to the antagonist.

The profile shown is typical of results obtained for repeated stimulation with nicotine (1 μM) in normal perfusion medium.

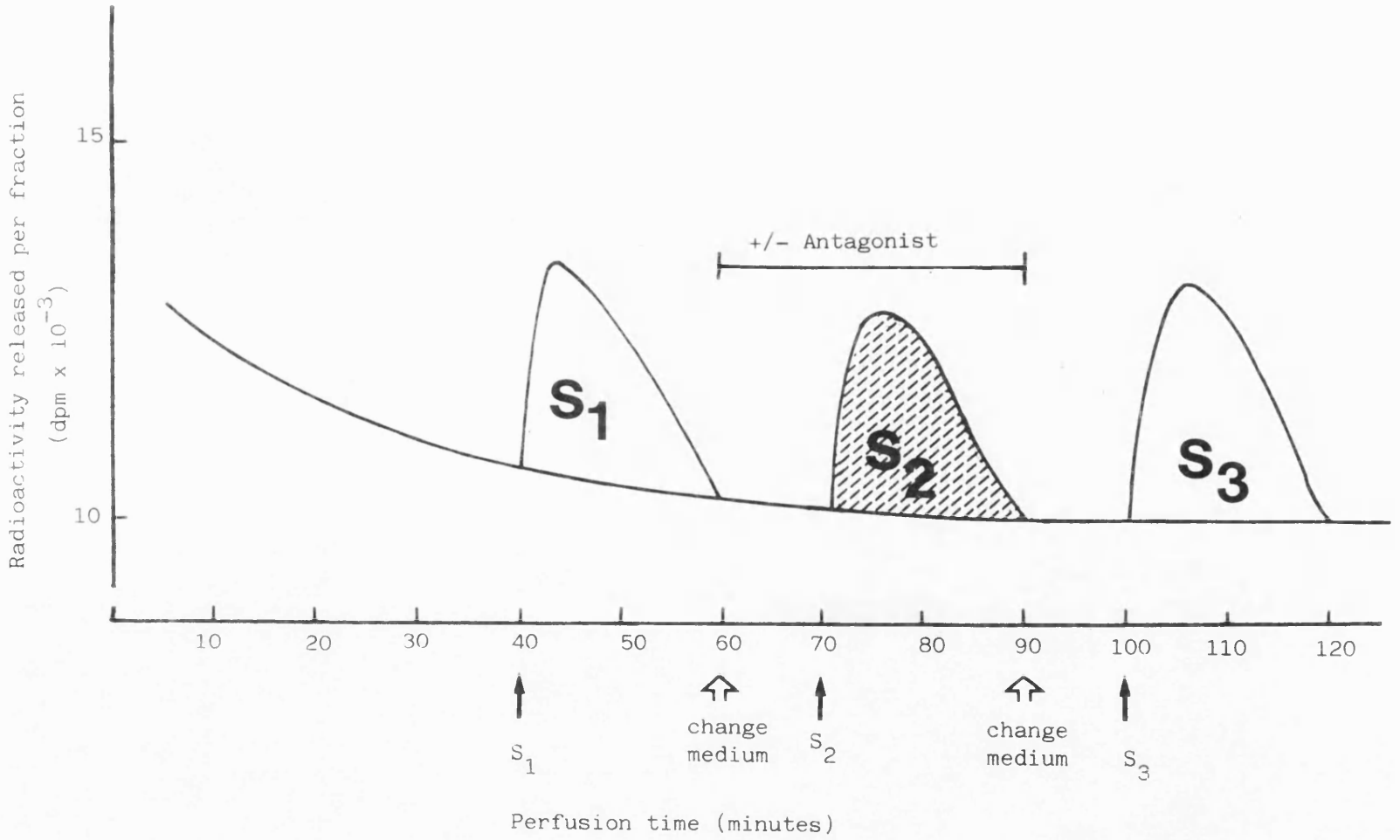


Fig. 3.14. The final perfusion schedule.

The test (antagonist present during S_2) and the control S_2/S_1 and S_3/S_1 values were then compared.

3.3. CHARACTERISATION OF THE FINAL SYSTEM

3.3.1. Determination of the possible reuptake of released [^{3}H]DA

A requirement of the perfusion system was that the [^{3}H] DA released from the preloaded synaptosomes was removed sufficiently quickly to prevent any reuptake. As previously determined, little binding of [^{3}H] DA to glass fibre filters occurs (Table 3.3).

The possible interaction of released [^{3}H] DA with the striatal synaptosomes was investigated by perfusing synaptosomes that had not been loaded and exposing them to pulses (100 µl) of [^{3}H] DA at a concentration approximately twice the amount estimated to be usually released in response to K^+ (28 mM), (1.1×10^{-9} M, 1.1×10^4 dpm/100 µl, see p. 165). However, this was an underestimate of endogenous DA levels which were later determined (see Section 3.3.3). A further pulse of K^+ (28 mM) was administered 25 min later to release any radioactivity that had been taken up by the synaptosomes. The effect of the DA uptake inhibitor nomifensine (10 µM) was determined by the continual perfusion of the drug in a parallel system.

Fig. 3.15 shows the distribution of the radioactivity after passage of a 100 µl pulse of [^{3}H] DA through the perfusion system in the presence and absence of synaptosomes. In both conditions the peak of tritium is distributed over 6 fractions which is equivalent to a volume of 2.04 ml and a time of about 13 min 10 s. This represents the diffusion of a pulse and was considered to be the minimum time required between successive stimulations. Analysis of the radioactivity remaining on the filter (Table 3.8) showed that in the presence of synaptosomes, four times as much [^{3}H] DA was retained than by the filters alone. Stimulation with K^+ failed to release the retained [^{3}H] DA suggesting either binding of the radiolabel to the

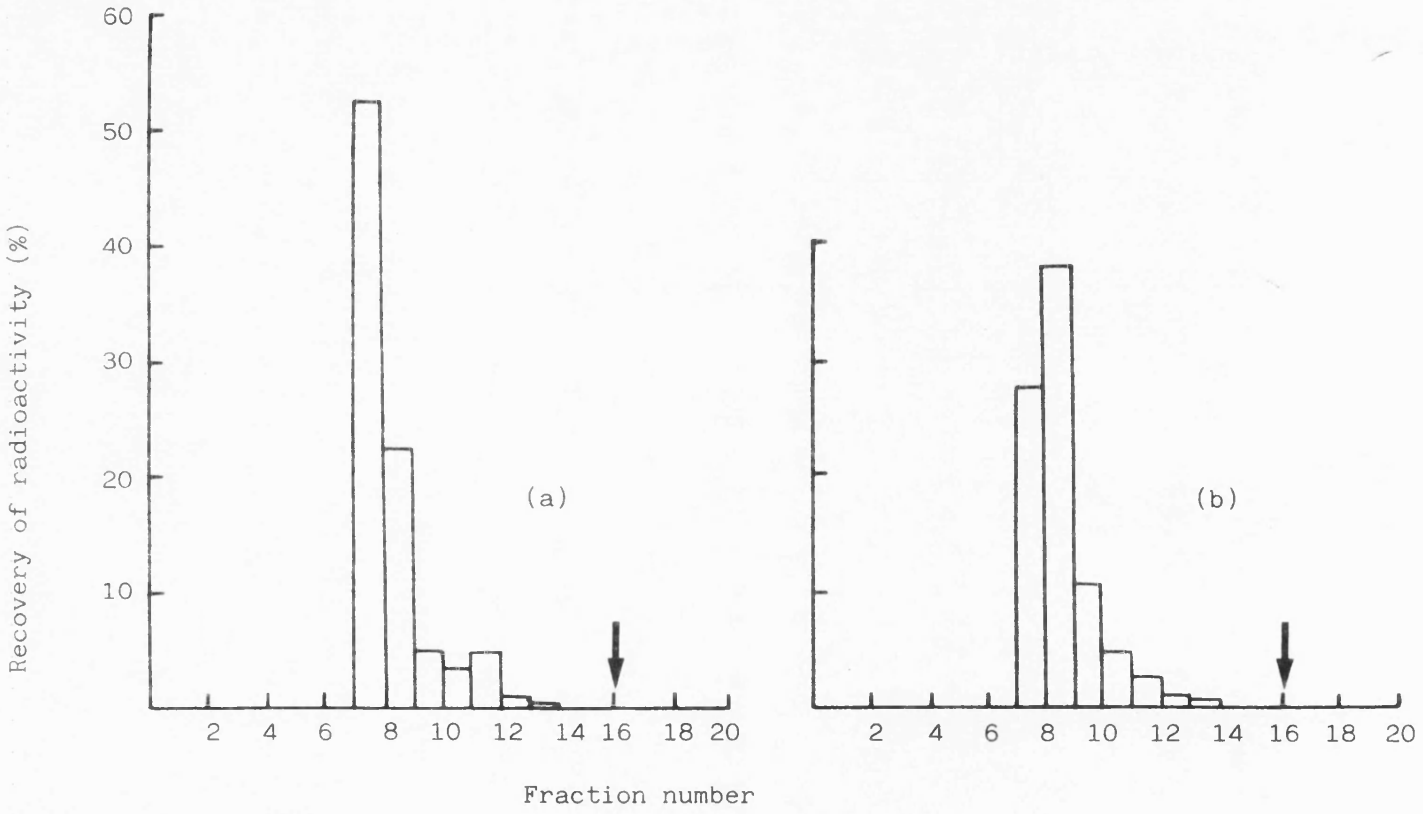


Fig. 3.15. Distribution of a 100 µl pulse of [^{3}H] DA (1.1 x 10^{4} dpm) using the final perfusion conditions (p.137)

a) Filters alone. b) Filters and synaptosomes (150 µl, 0.315 mg protein)

↓ indicates pulse of K^{+} (28 mM).

Table 3.8. Radioactivity remaining on filters after perfusion of a pulse of [^{3}H]DA (1.1 x 10^{4} dpm).

Results from a single experiment.

Perfusion details	Radioactivity remaining cpm	% pulse	fmoles DA retained
a) Filters alone	133	3.6	2.8
b) Filters with synaptosomes	466	12.7	11.2
c) Filter with synaptosomes. Perfusion in presence of nomifensine (50 µM)	457	12.5	11.0

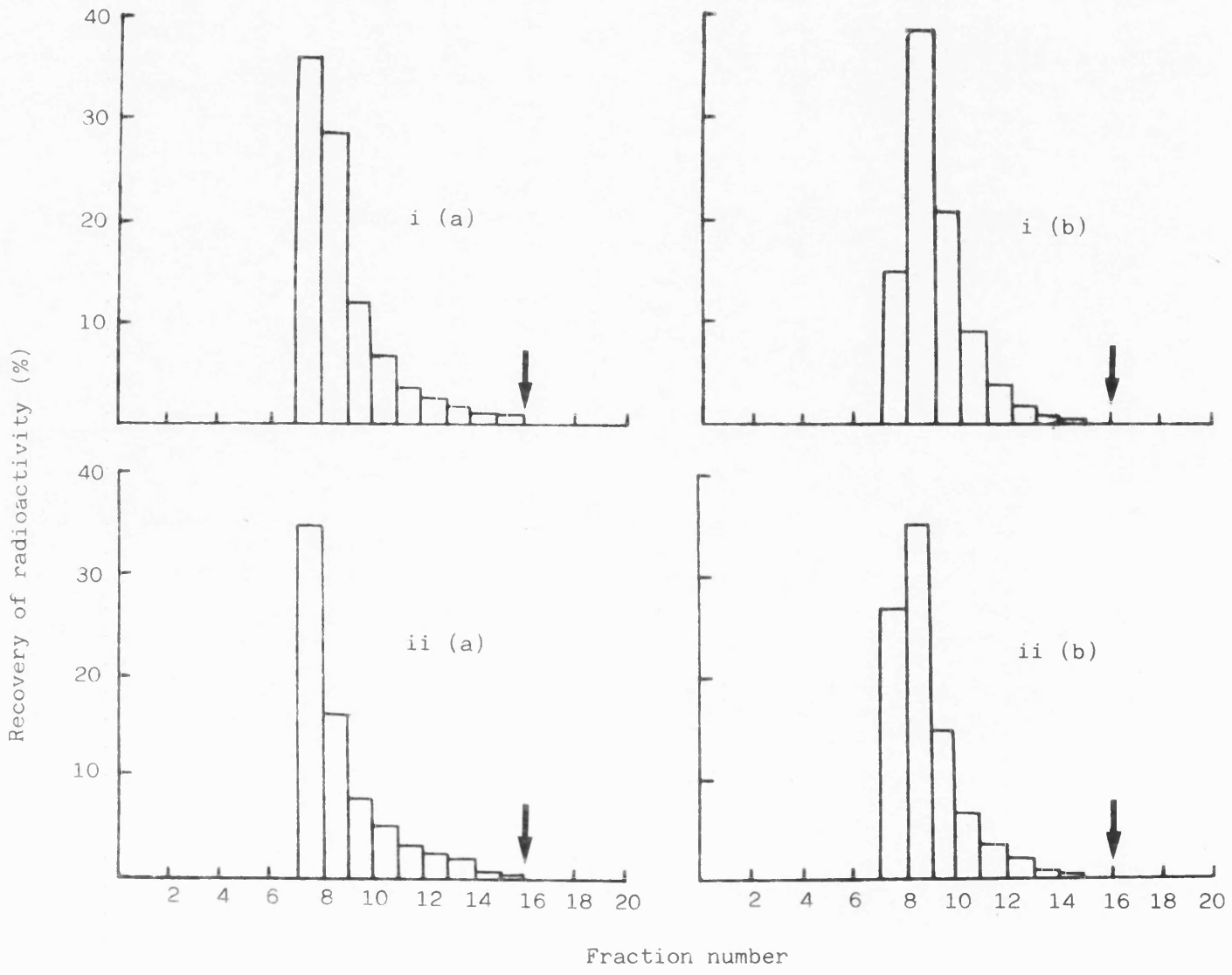


Fig. 3.16. Distribution of a 100 µl pulse of [^{3}H] nicotine using the final perfusion conditions (p 137)

(i) 10 µM Nicotine (3.3 x 10^5 cpm/100 µl) (ii) 1 µM Nicotine (3.3 x 10^4 cpm/100 µl)

(a) filters alone, (b) filters and synaptosomes (0.54 mg protein). ↓ indicates pulse of K^+ (28 mM).

Table 3.9. Radioactivity retained by the filters and synaptosomes after perfusion with a pulse of [^{3}H]nicotine. Results from a single experiment.

Concentration of nicotine	Perfusion details	Radioactivity remaining (cpm)	fmoles nicotine	fmoles nicotine associated with synaptosomes	fmoles nicotine /mg synaptosomal protein
(i) 10 μM (3.3×10^{5} cpm /100 μl)	with synaptosomes (b)	8803	446	260	481
	filters alone (a)	3664	186	-	-
(ii) 1 μM (3.3×10^{4} cpm/100 μl)	with synaptosomes (b)	6699	340	114	211
	filters alone (a)	4445	226	-	-

tissue or that uptake may have been into non-releasable pools. However, the DA uptake inhibitor nomifensine (10 μM) had no effect on the distribution of radioactivity either released or retained (profiles not included because they were identical to control), which strongly suggests that no specific uptake had occurred.

3.3.2. Determination of nicotine binding to glass fibre filters and tissue during a perfusion experiment

Nicotine has been reported to bind to glass fibre filters with high affinity (Abood *et al.*, 1979). To determine whether significant binding of nicotine to the filters and synaptosomes occurs during a perfusion experiment, synaptosomes were prepared and perfused as described in section 3.3.1. Pulses of [^{3}H]nicotine (29.8 Ci/mmol) were administered at two concentrations, 1 μM and 10 μM. Control systems were operated in parallel in the absence of synaptosomes.

Fig. 3.16 and Table 3.9 summarise the results obtained. The radioactivity was distributed over 6 to 7 fractions, similar to the diffusion profiles obtained with the pulses of [^{3}H]DA (Section 3.3.1, Fig. 3.15). Residual binding of the ligand to the filters was the same at the two nicotine concentrations studied. In contrast, in the presence of synaptosomes approximately twice the amount of nicotine was associated with the synaptosomes at the higher concentration (10 μM).These results support the decision to use low (1 μM) concentrations of nicotine to reduce the amount of residual nicotine binding between successive stimulations.

3.3.3. Analysis of the perfusate by HPLC with ECD.

To determine the nature of the released radioactivity and to

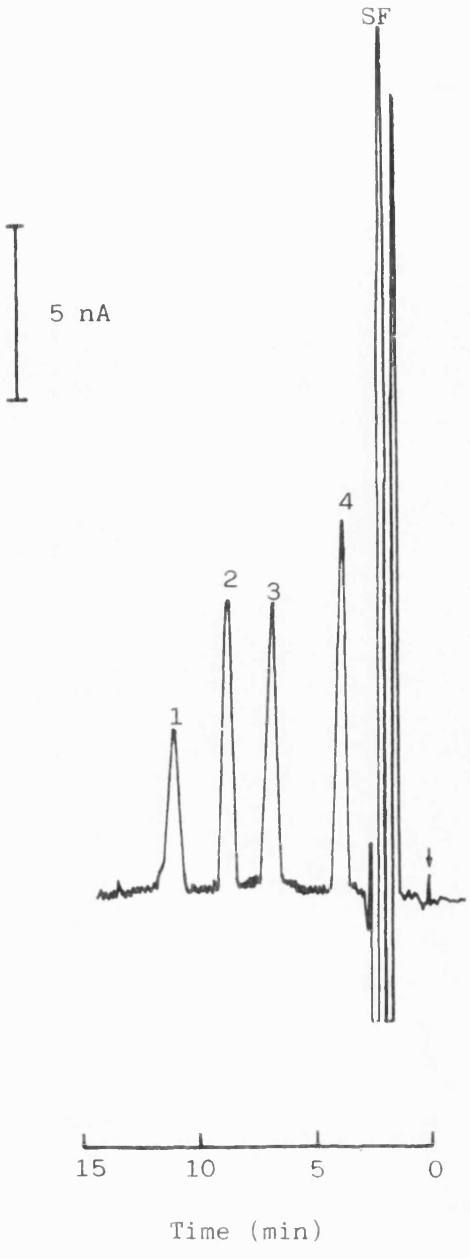


Fig. 3.17. A typical HPLC chromatogram obtained from a standard solution containing 10 pmoles/100 µl of (i) dopamine, (2) homovanillic acid, (3) dihydroxybenzylamine and (4) 3, 4- dihydroxyphenylacetic acid. Solvent front (SF). Arrow indicates time of injection.

estimate the levels of unlabelled (endogenous) DA, analysis of the perfusate by High Performance Liquid Chromatography with electrochemical detection (HPLC with ECD) was carried out.

The HPLC method was based on the chromatographic procedure described by Eriksson and Persson, (1982).

a) The HPLC system

The liquid chromatography system was composed of an LDC model III constametric pump, a Rheodyne 7125 injection valve with a 100 µl loop, a 20 cm stainless steel analytical column packed with 5 µm diameter Hypersil octadecylsilane (ODS) particles, and an electrochemical detector BAS LC-4A. The detector was operated at +0.65 V with a Ag/AgCl reference electrode (BAS RE-1) and a glassy carbon working electrode.

The mobile phase was an acetate-citrate buffer (pH 5.2) containing sodium acetate (100 mM), sodium hydroxide (60 mM), citric acid (40 mM), 1-octane sulphonic acid, sodium salt (0.5 mM) and methanol (HPLC grade, 10% v/v). The water used was deionised and filtered through a Millipore Milli-Q-reagent grade water system. Before use the mobile phase was filtered through a 0.45 µm pore nitrocellulose filter under vacuum and degassed by bubbling with helium for 30 min.

b) Operation and calibration of the column

The mobile phase was pumped through the column at a flow rate of 1 ml/min. A pressure of 1500 p.s.i. was maintained throughout the procedure and ambient temperatures were used. To demonstrate that DA and its metabolites could be resolved using the described system, fresh solutions (1 µM) of DA, DOPAC, homovanillic acid (HVA) and the internal standard dihydroxybenzylamine (DHBA) were prepared in

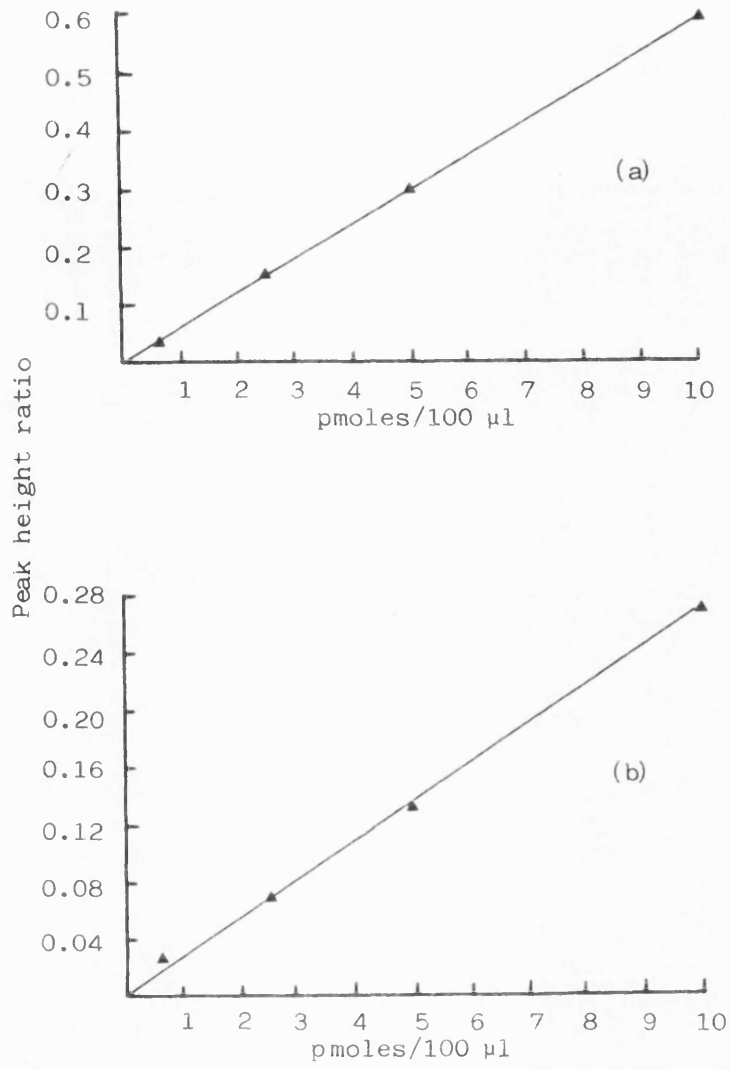


Fig. 3.18 Calibration curves for a) DOPAC and b) DA

Standard solutions (listed in the Table 3.10 below) were analysed in triplicate and the mean peak height ratio calculated.

a) DOPAC : $y = 0.0586\ x + 0.0047$ ($r = 0.999$)

b) DA : $y = 0.0261\ x + 0.0055$ ($r = 0.996$)

Table 3.10

Standard	pmoles in each 100 µl		
	DA	DOPAC	DHBA
1	0.625	0.625	20
2	2.5	2.5	20
3	5	5	20
4	10	10	20

Millipore-filtered water and a sample (100 µl) of each solution injected into the column. The sensitivity of the ECD was set at 5 nA and the retention time determined for each test compound (Table 3.11). The retention time was dependent on daily variables such as room temperature and the percentage methanol composition of the mobile phase.

Table 3.11. The characteristic retention time of each compound studied using the HPLC system

Compound	Retention time min, sec
DOPAC	3, 55
DHBA	6, 45
HVA	8, 30
DA	10, 45
Solvent front	2, 0

Because the retention time of DHBA did not overlap with that of any of the other test compounds (Fig. 3.17) it was a suitable internal standard. DHBA, at a constant concentration, 20 pmoles/100 µl, was added to each sample to be analysed, as an internal standard to correct for variation in sample injection size and manipulation. The ratio of the peak height of the test compound to the height of the DHBA peak on the HPLC chromatogram was used to calculate the concentration of the test compound by reference to calibration curves (Fig. 3.18). This is known as the peak height ratio.

c) Analysis of [^{3}H]DA by HPLC with ECD

To determine the purity of [7,8-^{3}H]DA used in the perfusion experiments, a 100 µl sample of [^{3}H]DA, diluted with Millipore-filtered water, to a concentration of 2 x 10^{-8} M, was injected into the HPLC column and 30 s fractions (0.5 ml) of the eluate collected in scintillation vials at the waste pipe from the E.C.D. The radioactivity in each fraction was measured (p.**74**) and expressed as a percentage of the total radioactivity recovered from the column, corrections for background counts and counting efficiency being made. The time taken from the ECD to the waste pipe was 30 s, hence the retention time for each peak of radioactivity could be determined and the tritiated compound identified. Two samples of [^{3}H]DA were analysed; sample a) which had been stored for 18 months and sample b) for 3 months. Analysis of the distribution of tritium indicated that a large amount of decomposition had occurred in the older sample, with unidentifiable products eluting with the solvent front (Fig. 3.19). The HPLC chromatograms (not included) had horizontal baselines characteristic of clean samples. Using a sensitivity of 5 nA a single peak at t = 11 was obtained for each sample, which correlates well with the expected position of DA (Fig. 3.17).

d) Extraction of DA

The extraction of DA from aqueous samples was carried out following the method of Smedes *et al.* (1982), with the modifications by Ian MacDonald (personal communication).

To a sample (1 ml) of perfusate or standard solution, in a glass test tube, 0.5 ml of 2 M NH_4Cl-NH_4OH buffer (pH 8.5) containing 0.2% (w/v) diphenylborate-ethanolamine (DPBEA) and 0.5% (w/v) EDTA was

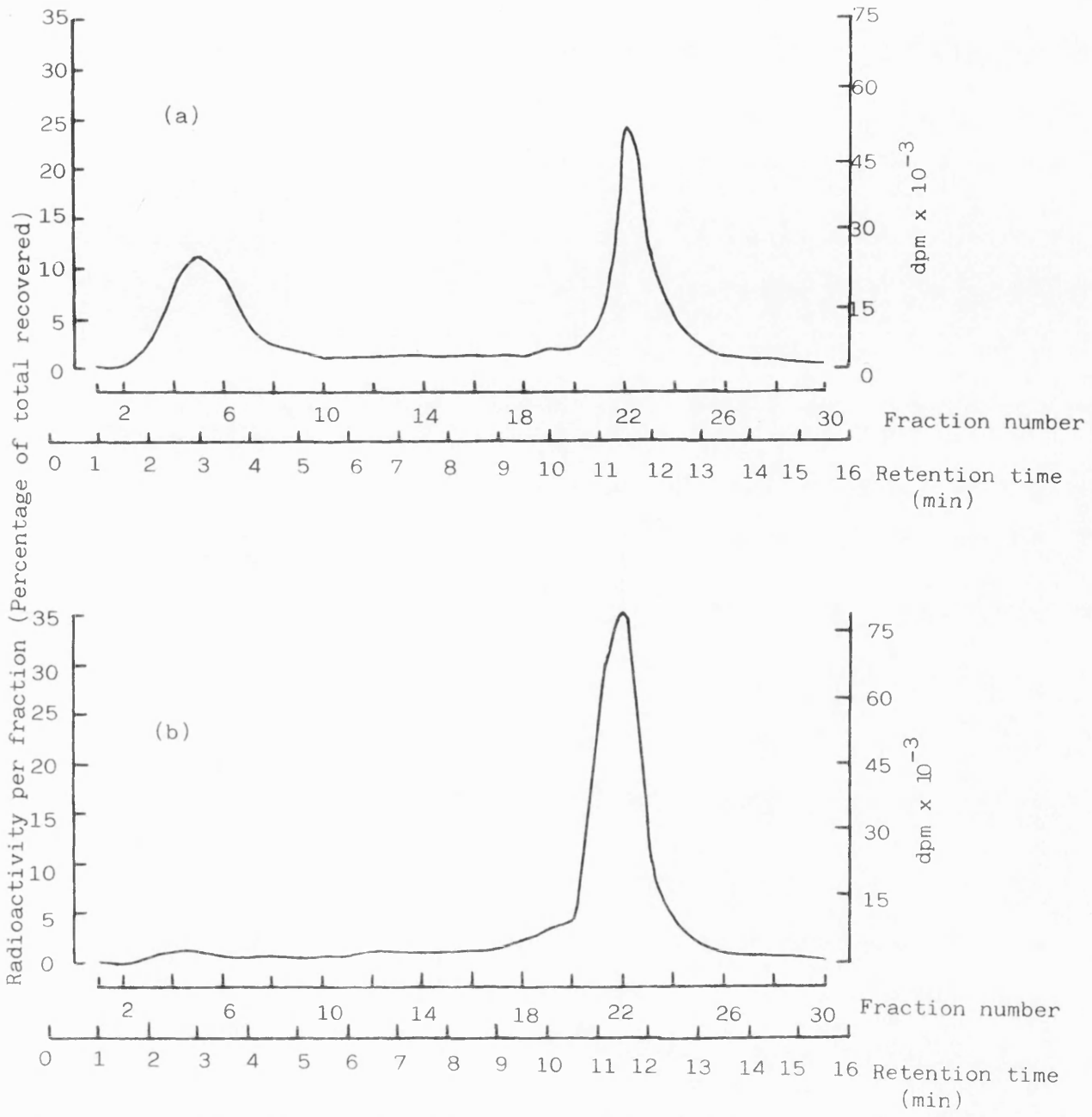


Fig. 3.19 Distribution of tritium in samples of radiolabelled dopamine (2 pmoles/100 µl) stored as described in "Methods".

Sample a) 18 months old

Recovery of radioactivity = 99.5%

Tritium associated with DA = 44%

Sample b) 3 months old

Recovery of radioactivity = 96.5%

Tritium associated with DA = 77.5%

added followed by 2.5 ml of n-heptane containing 1% (v/v) octanol and 0.35% (w/v) tetraoctylammonium bromide (TOABr). The mixture was vortexed for 2 min and then left for 5 min at 4°C to allow the organic and aqueous layers to separate. A portion of the organic layer (2 ml) was transferred to a glass centrifugate tube. Reversion to the aqueous phase was achieved by addition of 1 ml octanol and 250 µl 0.08 M acetic acid. The mixture was vortexed for 1 min and then left for 5 min to allow separation of the layers. Using a Pasteur pipette the lower aqueous phase was removed and stored in an Eppendorf tube at 4°C until analysis by HPLC with ECD.

To determine the concentration of the DA in the acetic acid, the internal standard (DHBA) was included in the 0.08 M acetic acid (20 pmol DHBA/100 µl) and the peak-height ratio calculated.

e) Analysis by HPLC with ECD of the perfusate released from striatal synaptosomes

A suspension of striatal synaptosomes was prepared (p.**63**) and divided into two. To one half [^{3}H]DA was added, using the loading conditions described in Section 2.3.5 (p.**95**). The remaining sample was incubated at 37°C for an equivalent incubation period. Samples (200 µl) of the synaptosomes were layered onto the filters in the perfusion chambers. Three chambers were operated in parallel, two containing tissue preloaded with [^{3}H]DA and one with unloaded tissue. Perfusion was carried out using the final conditions (p.**137**) except that a longer washout period of 60 min was used before exposure to a pulse of test substance, and larger fractions (30 drops, 1.35 ml) were collected. The fractions containing tritium were analysed as follows:

1) The radioactivity in a sample (50 µl) from each fraction was counted (p.74) to give a perfusion profile (Fig. 3.20).
2) 100 µl from each of the 3 fractions around the peak of stimulation was injected into the HPLC column and 1 min fractions of the eluate collected from the waste pipe of the ECD. The fractions were counted for radioactivity (p.74) and the distribution of tritium determined (Fig. 3.20; lower profiles).
3) Samples (1 ml) from the 3 fractions around the peak of stimulation were extracted using the method described on p.159. 100 µl portions of the final extracts, containing 20 pmoles DHBA/100 µl 0.08 M acetic acid, were counted for radioactivity (p.74) and analysed by HPLC with ECD (Table 3.13).

Fractions containing DA released from the unloaded synaptosomes were extracted by the method described in Section 3.3.3d and the extract analysed by HPLC with ECD (Table 3.14).

To determine the effectiveness of the extraction process three samples were compared,

i) an extract from a standard solution originally containing 10 pmoles DA, 10 pmoles DOPAC and 20 pmoles DHBA per 100 µl;
ii) an extract from a standard solution containing 10 pmoles DA/100 µl, the internal standard DHBA (20 pmoles/100 µl) added to the final extract;
iii) a standard solution containing 10 pmoles DA and 20 pmoles DHBA per 100 µl (no extraction); see Table 3.12.

The peak-height ratios of DA relative to DHBA were similar for samples i) and iii), indicating that DHBA and DA were equally ex-

tracted. Calculation of the DA concentration in sample (ii), by extrapolation of the calibration curve, gave a concentration of greater than 20 pmoles DA/100 µl indicating that the extraction method produced a two-fold increase in the concentration of DA. Inclusion of DOPAC in the standards (i) gave no additional peak on the chromatogram, indicating that DOPAC was not extracted.

Table 3.12. Comparison of extracted DA/DHBA samples by HPLC.

Sample	Peak-height ratio	Approx. DA concentration pmoles/100 µl
i)	0.267	10
ii)	0.633	24
iii)	0.250	9.4

Results are the means of three determinations, range in peak-height ratio ± 0.02.

Table 3.13. The concentration of [^{3}H]DA in samples of the perfusate fractions from perfusion system a) (K^+ stim., Fig. 3.20a) was calculated by reference to the specific activity of the radiolabel (10^5 dpm/pmole)and the percentage of the tritium associated with DA.

$$\frac{\text{dpm/100 µl}}{10^5} \times \% \text{ DA} = \text{pmoles } [^3\text{H}]\text{DA/100 µl}$$

The efficiency of the extraction process was determined by comparing the concentration of [^{3}H]DA in the extracted samples with the untreated fractions. The results showed that a correction factor of 2.15 (mean of 3 samples) was required to take account of the concentration effect of the extraction process.

Table 3.13. Estimation of the concentration of [^{3}H]DA in perfusate samples.

Fraction	dpm/100 µl	% dpm DA	% dpm DOPAC	pmoles [^{3}H]DA /100 µl	Increase in concentration compared with non-extracted samples
6A	4961	80.5	0	0.040	-
7A	6360	81	8	0.051 (∿ 0.5 nM)	-
8A	4706	83	0	0.039	-
Extracted samples					
6A'	8817	91	0	0.080	x 2
7A'	12483	94	0	0.117	x 2.29
8A'	9017	93	0	0.084	x 2.15

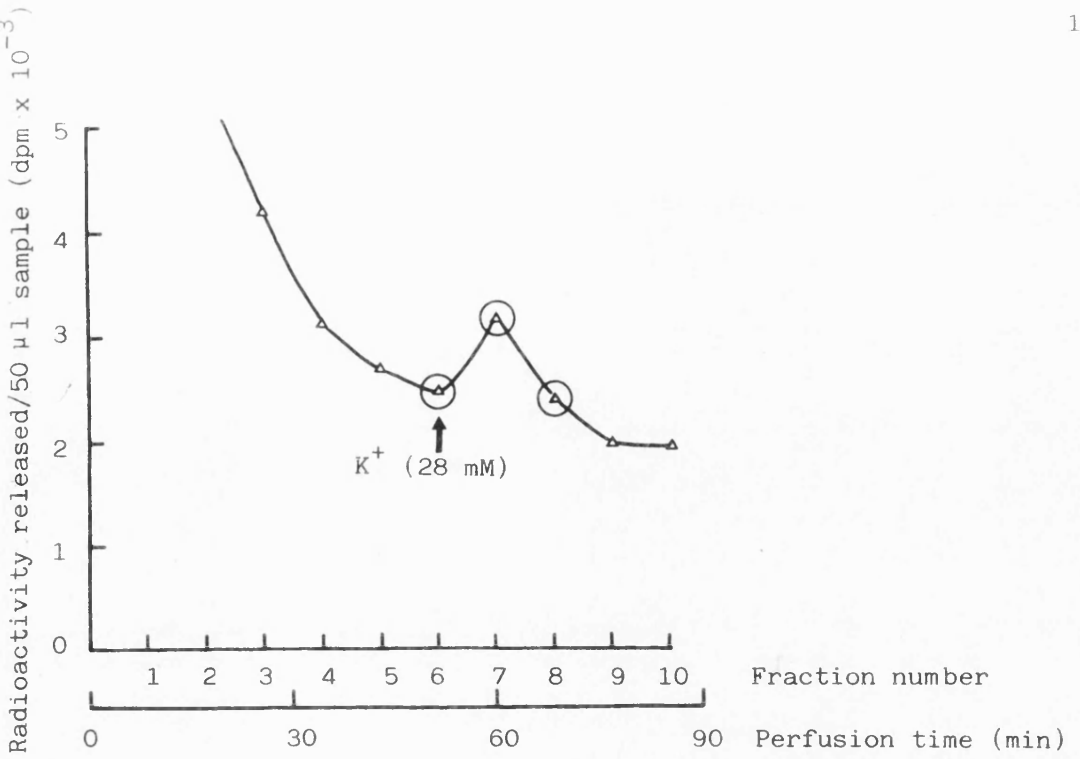
Table 3.14. Measurement of endogenous DA

Fraction Number (see Fig. 3.20) extracted sample	Peak height ratio	pmoles /100 µl	Estimated DA concentration (nM)
5	0.025	0.346	1.7
7	0.03	0.432	2.2
9	0.024	0.329	1.6

The perfusate released from the unloaded synaptosomes was analysed by HPLC with ECD after extraction of the fractions by the method described on p.**159** . Small peaks with a retention time of 11 min were observed on the HPLC chromatograms. Using the peak-height ratio, the concentration of DA in the extracted samples was estimated and the true level of DA in the non-extracted samples determined, (assuming the doubling of the DA concentration in the final extract; Tables 3.12 and 3.13).

Fig. 3.20. Analysis of the [^{3}H]DA released from perfused striatal synaptosomes in response to a) K^{+} (28 mM) and b) nicotine (1 μM).

Upper profiles show the radioactivity present in 50 μl samples of consecutive fractions (1.3 ml). The lower profiles show the separation by HPLC of the tritium in 100 μl samples of the 3 fractions around the peak of stimulation (6, 7, 8). Recovery of radioactivity from the HPLC column was approximately 80%. In response to K^{+} and nicotine stimulation (fractions 7a and 7b respectively), 8% of the radioactivity recovered was associated with a peak of retention time characteristic of DOPAC (shaded area). The other peak of tritium with a retention time of 11 min was attributed to DA.


Radioactivity released/50 µl sample (dpm x 10^{-3})
0
1
2
3
4
5
K^+ (28 mM)
1 2 3 4 5 6 7 8 9 10
Fraction number
0
30
60
90
Perfusion time (min)

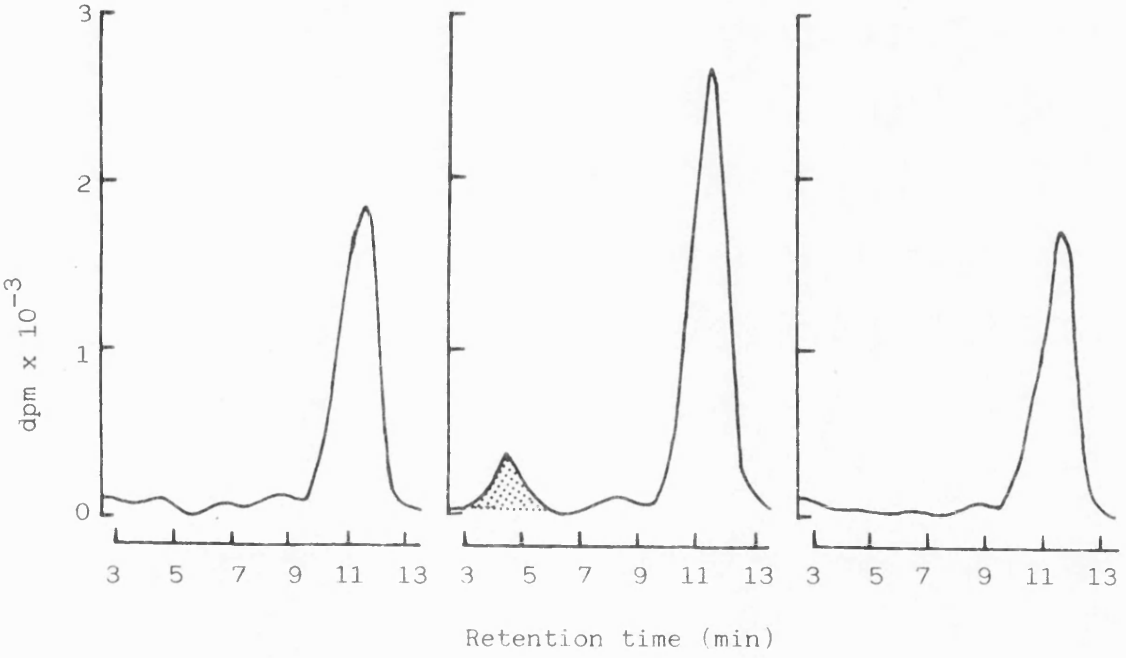
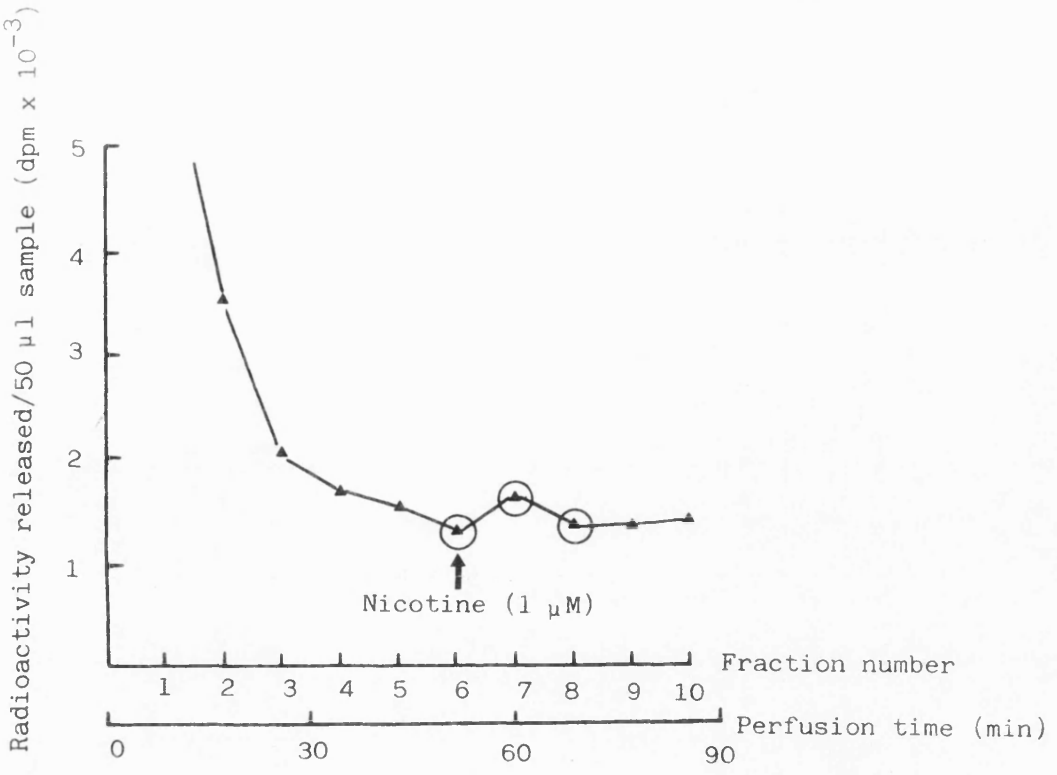

dpm x 10^{-3}
0
1
2
3
3 5 7 9 11 13
3 5 7 9 11 13
3 5 7 9 11 13
Retention time (min)

Fig. 3.20a.


Radioactivity released/50 µl sample (dpm x 10^{-3})
5
4
3
2
1
Nicotine (1 µM)
Fraction number
1 2 3 4 5 6 7 8 9 10
Perfusion time (min)
0
30
60
90

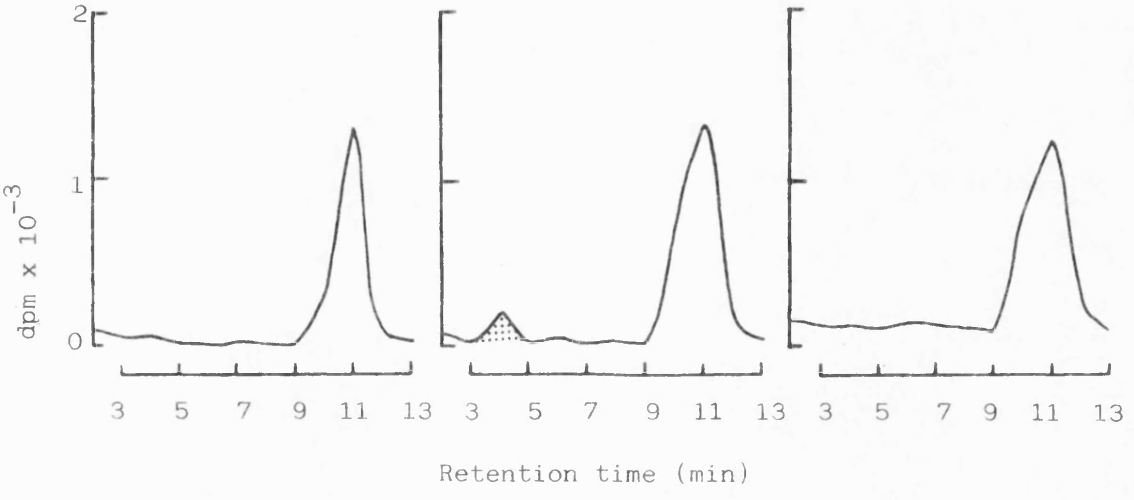

dpm x 10^{-3}
2
1
0
3 5 7 9 11 13 3 5 7 9 11 13 3 5 7 9 11 13
Retention time (min)

Fig. 3.20 b.

f) Discussion: Analysis of DA by HPLC with ECD

The HPLC system used, that of reverse phase ion-pair chromatography, allowed the separation of DA from its metabolites (Fig. 3.17). However, the usefulness of the system was limited by the detection limit of the ECD (0.5 pmole DA/100 µl). Furthermore, because the concentration of DA in the perfusate was low (~ 0.5 nM [^{3}H]DA and ~ 2 nM DA) (Tables 3.13 and 3.14), accurate measurement of the peaks on the chromatogram was difficult. The HPLC system was therefore initially used to separate samples containing tritium and to analyse the eluate from the column for radioactivity. Using this approach the purity of stock [^{3}H]DA was determined; a 3 month old sample (stored as described in the "Methods") had a purity of 77.5% (Fig. 3.19). The period of storage is therefore important. Stabilising agents such as ascorbic acid are often added, but this has been shown to produce a front peak on the chromatogram (Eriksson *et al.*, 1982). The importance of refrigeration, storage in the dark and the addition of perchloric acid (0.1 M, to deproteinise the sample) have also been used to minimise degradation of DA (Eriksson *et al.*, 1982 ; Verbiese-Genard *et al.*, 1983). To ensure minimum degradation of the DA used in subsequent experiments [^{3}H]DA was stored for a maximum of 3 months in 2.5 mM ascorbic acid at -20°C.

The tritium present in the perfusate was assumed to be [^{3}H]DA. The major route of DA metabolism in striatal synaptosomes is by deamination to form DOPAC (de Belleroche *et al.*, 1976). However, little degradation of the released [^{3}H]DA was expected because of the presence of the MAO inhibitor pargyline (10 µM) and the anti-oxidant ascorbic acid (1 mM) in the perfusion medium. Pargyline

inhibits the breakdown of DA to form DOPAC by inactivating MAO whereas ascorbic acid prevents oxidation of DA by atmospheric oxygen.

Many groups who have studied the release of DA from striatal synaptosomes have included MAO inhibitors and ascorbic acid at concentrations similar to those used in the experiments in this thesis (e.g. Raiteri *et al.*, 1974, 1979; Mulder *et al.*, 1975; Minnema and Michaelson, 1985). Indeed, their presence has been shown to reduce the basal efflux of the primary metabolite DOPAC from perfused striatal slices (Becker *et al.*, 1984; Arbuthnot *et al.*, 1984) and, when MAO is not inhibited, the spontaneous outflow of radioactivity consists primarily of [^{3}H]DA metabolites (Cubeddu *et al.*, 1979).

Using perfusion medium containing pargyline and ascorbic acid, approximately 80% of the released tritium had a retention time characteristic of DA (Fig. 3.20), suggesting that little degradation had occurred. The purity of the stock [^{3}H]DA used to radiolabel the synaptosomal transmitter pools was also about 80% (Fig. 3.19) and so the original assumption that the released radioactivity was mainly [^{3}H]DA was correct. Of particular interest was the finding that no [^{3}H]DA metabolites were detected in the fractions before and after stimulation, but during K^+ (28 mM) and nicotine (1 μM) evoked release an additional peak of tritium was observed characteristic of DOPAC (Fig. 3.20). The DOPAC released upon stimulation is possibly formed from DA released from the storage granules into the cytoplasm where it is accessible to MAO (Cubeddu *et al.*, 1979). This also agrees with the work of Arbilla and Langer (1978) who suggest that K^+-evoked release causes the

efflux of [^{3}H]DA from reserpine-sensitive stores (storage granules).

In a preliminary experiment, the omission of pargyline resulted in a low uptake of radiolabel, corresponding to a reduced level of basal efflux (20% normal) and small peaks of evoked radioactivity. Despite the reported criticisms of the use of MAO inhibitors and ascorbic acid in release studies (Hadjiconstantiou and Neff, 1983; Arbilla and Langer, 1980; Collard *et al.*, 1980), pargyline (0.01 mM) and ascorbic acid (1 mM, a concentration half that used by Mills and Wonnacott, 1984) were always included in the perfusion medium. This was to ensure a high uptake of [^{3}H]DA which was required for the improved sensitivity of the final perfusion system.

To measure the endogenous levels of the released DA, the extraction method of Smedes *et al.* (1982) was used. The procedure makes use of the complex formation in alkaline medium, between the diphenylborate and the diol group of the catecholamines in combination with ion-pair formation. The method did not extract DOPAC and therefore no information about the endogenous levels of this metabolite was obtained. DA and the internal standard DHBA were extracted with similar efficiency (Table 3.12). Using standard solutions, the extraction method approximately doubled the concentration of DA (Table 3.13). The concentration of DA in the final extract was at a level which was just above the detection limit of the ECD; the endogenous level was calculated as approximately 2 nM, giving a ratio of labelled to unlabelled DA of 1 : 4.

3.3.4 The physiological relevance of the released [^{3}H]DA

a) Basal release

The release of transmitter from perfused tissue, in the absence of stimulating agents, is often referred to as the basal or spontaneous efflux/outflow.

Perfusion of a tissue preparation allows the study of both basal and evoked release. For a synaptosomal preparation preloaded with radiolabelled transmitter precursor, high levels of basal release can lead to difficulties in studying the release mechanisms (Mulder, 1982). Basal efflux may represent leakage from damaged particles, although efflux from ruptured nerve endings has no physiological relevance but is one of the disadvantages of using a synaptosomal preparation to study transmitter release (Levi and Raiteri, 1976). The amount of basal release is dependent upon the amount of tissue and on the integrity of the preparation. Residual radiolabelled transmitter which has not been taken up by the synaptosomes can also contribute to the basal level of radioactivity. Using the final perfusion conditions (p.**137**) a stable basal rate of release was established after 40 min. Similar washout periods have been used by other groups (e.g. Mulder *et al.*, 1975; Taube *et al.*, 1977 and Gillet *et al.*, 1985).

A Ca^{2+}-dependent component of the basal release was demonstrated by initially perfusing striatal synaptosomes with Ca^{2+}-free medium (p.**137**) and then introducing Ca^{2+} into the medium (Fig. 3.21). There was a marked increase in the spontaneous release of [^{3}H]DA upon the addition of Ca^{2+}. A similar Ca^{2+}-dependent component of basal release of radiolabelled transmitter from perfused synaptosomes has been reported by Raiteri *et al.* (1979);

Minnema and Michaelson (1985). However, there remained a large proportion of the basal efflux which was Ca^{2+}-independent. This may represent efflux from non-vesicular transmitter stores (Mulder *et al.*, 1975), or the non-quantal spontaneous release reported by Katz and Miledi (1977). It may also represent efflux from damaged synaptosomes. Alternatively, the observed Ca^{2+}-independent release may be a result of high levels of endogenous Ca^{2+} present in the synaptosomal preparation. Examination of the synaptosomal fraction by electron microscopy (Fig. 2.3, p.78) showed that free mitochondria were present in the preparation. These Ca^{2+}-rich organelles may therefore supply the Ca^{2+} required for transmitter release.

In an attempt to obtain a complete Ca^{2+}-free condition, perfusion of striatal synaptosomes was carried out using Ca^{2+}-free medium containing 0.5 mM EGTA. However, after 40 min perfusion with this medium and one stimulation with 0.01 µM nicotine, there was a massive continual release of transmitter which levelled off at a basal rate twice the control value. Further studies using EGTA were not carried out because of this observed effect on the basal release.

b) The Ca^{2+}-dependency of K^+-evoked [^{3}H]DA release

To demonstrate the Ca^{2+}-dependency of K^+-evoked release of [^{3}H]DA from perfused striatal synaptosomes, preloaded striatal synaptosomes were initially perfused with Ca^{2+}-free medium (p.137) and then with normal perfusion medium containing 2.5 mM Ca^{2+}. Two systems were operated in parallel, one serving as a control to monitor changes in basal release rates and the other as the test system.

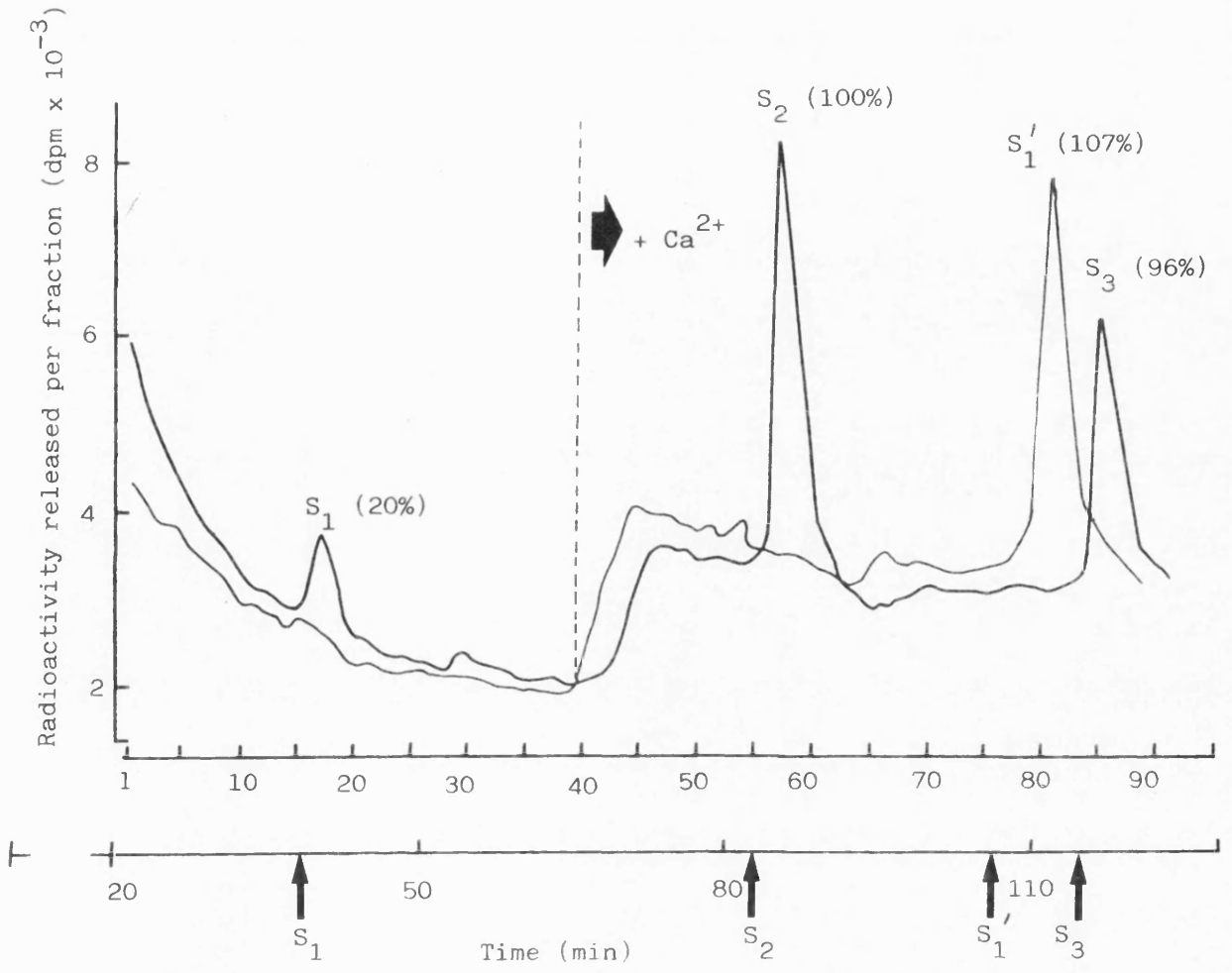


Fig. 3.21 Ca^{2+}-dependency of K^+-evoked release of [^{3}H]DA from striatal synaptosomes

Preloaded striatal synaptosomes (250 µl) were perfused using the final conditions (p.137) and small (4 drop) fractions were collected. The synaptosomes were initially perfused with Ca^{2+} free medium and then with normal medium (after dotted line). Pulses of K^+ (28 mM) were administered in the presence and absence of Ca^{2+} at the times indicated (↑). Two systems were operated in parallel, test system (heavy line) and control (light line).

The evoked release of [^{3}H] DA by K^+ (28 mM) in the absence of Ca^{2+} was 20% of the release value (S_2) obtained when Ca^{2+} was present.

The evoked release by K^+ (28 mM) in the absence of Ca^{2+} was 20% of the release obtained when Ca^{2+} was present (Fig. 3.21). The perfusion profiles obtained from the test and control systems clearly demonstrated the return to normal response when Ca^{2+} was added to the medium; after 110 min perfusion both control and test systems gave comparable responses (107% and 96% of the first K^+ pulse in Ca^{2+} medium (S_2) respectively). Complete Ca^{2+} dependency was not expected because of the possibility of residual Ca^{2+} (as discussed above in the Ca^{2+} dependency of basal release). Indeed Minnema and Michaelson (1985), have shown that maximum transmitter release (evoked by K^+ depolarization) occurred at Ca^{2+} concentrations as low as 0.254 mM.

These results confirm the Ca^{2+} dependency of transmitter release evoked by depolarization (Cotman *et al.*, 1976; Rubin, 1982) and show that the perfusion system can clearly demonstrate this effect, an important criterion of physiological transmitter release.

c) The effect of tetrodotoxin on stimulated release of [^{3}H]DA from striatal synaptosomes

Using the synaptosomal perfusion system three different types of stimuli, nicotine, K^+ and veratridine had been shown to evoke [^{3}H]DA from striatal synaptosomes (Fig. 3.11, p.141). The alkaloid veratridine acts specifically at voltage-dependent sodium channels in such a way that the channels remain open and there is an increase in permeability to Na^+ (Ohta *et al.*, 1973; Ulbricht, 1969). The influx of Na^+ leads to membrane depolarization and enhanced Ca^{2+} permeability which initiates transmitter release. The

action of veratridine in eliciting transmitter release from the striatal synaptosomes is evidence for voltage-dependent sodium channels on the synaptosomes. The voltage-dependent sodium channel is normally involved in the generation and propagation of nerve impulses (Hodgkin and Huxley, 1952) along the nerve axon and has been studied using the specific blocking agent tetrodotoxin (TTX) (Kao, 1966).

Experiments were therefore carried out to determine the effect of continuous perfusion of TTX (0.5 μM) on [^{3}H]DA release evoked by the three different stimuli, veratridine, K^+ and nicotine.

Striatal synaptosomes preloaded with [^{3}H]DA were perfused continuously with either normal perfusion medium (p.**135**) or medium containing TTX (0.5 μM). After the 40 min washout period the synaptosomes were successively stimulated with nicotine (1 μM), K^+ (28 mM) and veratridine (10 μM; Fig. 3.11, p.**141**). The radioactivity released by each stimulus in the presence of TTX was expressed as a percentage of the normal response obtained in a parallel control system (Table 3.15). The inclusion of TTX (0.5 μM) had no effect on the basal rate of release but completely abolished the 10 μM veratridine evoked [^{3}H]DA release. The responses by 1 μM nicotine and 28 mM K^+ were unaffected by the presence of TTX.

Table 3.15. The effect of TTX (0.5 μM) on stimulated [^{3}H]DA release from striatal synaptosomes

Stimuli	Response in the presence of TTX (0.5 μM) (Percentage control)
Nicotine 1 μM	94.8
K^+ 28 mM	104.0
Veratridine 10 μM	3.2

Results expressed as mean with variance ± 10% (n > 4).

3.3.5 Discussion

The final perfusion system fulfilled the original aim to produce a sensitive synaptosomal perfusion procedure in which synaptosomes could be repeatedly stimulated with little reuptake of the released transmitter. A factor contributing to the improved sensitivity of the perfusion system was the use of [^{3}H]DA of high specific activity. However, the use of radiolabelled neurotransmitters in release studies has been criticised (Besson *et al.*, 1969). The radiolabelled neurotransmitters may label intracellular DA pools that are not readily releasable. The DA that is most recently released is also that which has most recently been taken up (Besson *et al.*, 1969). However, in view of the long duration of the perfusion experiments, the preloading of the striatal synaptosomes with [^{3}H]DA cannot be considered as a recent event. Despite the criticisms of the use of radiolabelled neurotransmitters, they are commonly used to study presynaptic events. Recent studies by Bonnano *et al.* (1985) have shown that the measurement of either endogenous DA or [^{3}H]DA previously taken up by striatal synaptosomes gives similar results when studying K^+-evoked transmitter release, supporting the use of radiolabelled transmitters. Indeed the HPLC studies described above showed that both endogenous DA and [^{3}H]DA, in the presence of pargyline, are released in response to stimulation. However, the amount of DA in the perfusate is low and it would be inaccurate (as well as impractical) to routinely measure the DA in each fraction using HPLC with ECD. The radiolabelling of the DA pools in striatal synaptosomes was therefore required for a detailed study of the nicotine regulation of DA release. [^{3}H]DA was selected instead of [^{3}H]tyrosine to avoid the

necessary separation of [^{3}H]dopamine from the perfusate. [^{3}H]DA is also of a higher specific activity than [^{3}H]tyrosine. In support of the use of [^{3}H]DA was the demonstration of the Ca^{2+}-dependency of both the basal and K^{+}-evoked release of tritium.

In contrast to the sensitivity of the veratridine-evoked release of [^{3}H]DA to TTX (which confirms that voltage-dependent sodium channels are present on striatal nerve terminals), the lack of effect of TTX on nicotine evoked release provides evidence that the nicotinic mechanism does not involve the voltage-dependent sodium channel. Using the same concentrations of nicotine and TTX, Giorguieff-Chesselet *et al.* (1979) obtained similar results using a perfused striatal slice preparation. However, contrary to the results reported in this thesis, Giorguieff *et al.* (1977) reported that the basal release was partially TTX-sensitive, indicating the possible involvement of interneurones. These results show that when using slice preparations it is important to carry out control experiments in the presence of TTX to abolish any excitatory effects produced by interneurones.

The agreement between the results of Giorguieff *et al.* (1979) and the results reported in this thesis is of interest because of the differences in the preparation and labelling methods. Giorgueff and coworkers continuously perfused striatal slices with [^{3}H] tyrosine and measured the release of newly synthesised [^{3}H]DA in contrast to the measurement of the release of newly taken up [^{3}H]DA from striatal synaptosomes. This suggests that in release studies either the precursor or the transmitter may be used to radiolabel the dopaminergic transmitter pools. Indeed, de Langen *et al.* (1979) reported that [^{14}C]DA synthesised within striatal synaptosomes from

[^{14}C]tyrosine is not released by K^+ depolarisation in preference to [^{3}H]DA previously taken up by the synaptosomes.

The similarity between the results reported in this thesis and those of Giorguieff *et al.* (1979) also confirms the validity of the synaptósomal perfusion technique. The advantages and disadvantages of a synaptosomal or slice preparation have been reviewed by Gibson and Blass (1982) and Weiler *et al.* (1982). A slice preparation more closely resembles the situation *in vivo*, although the measurement of presynaptic events is complicated by the presence of non-neuronal and neuronal interconnections. Synaptosomes are devoid of axons and cell bodies which may normally influence nerve activity. Another disadvantage of a synaptosomal preparation is that it is usually contaminated with mitochondria which could alter the levels of Ca^{2+} and hence the release of transmitter. Nevertheless, synaptosomes have several advantages including the ease and reproducibility of their preparation. They are also probably more sensitive to low concentrations of drugs and will not have the problems of diffusion associated with slice preparations (Wood and Sidhu, 1986).

CHAPTER 4

CHARACTERISATION OF THE NICOTINIC REGULATION OF STRIATAL DA RELEASE

4.1 INTRODUCTION

From the reports in the literature the nicotinic regulation of DA release in the striatum has been shown to be sensitive to both C_6 and C_{10} antagonists (see Table 1.2). The characterised perfusion system provides an appropriate method for a detailed pharmacological analysis of the nicotinic heteroreceptor mediating the release of [^{3}H] DA from striatal synaptosomes. Because of the problems associated with using ACh as an agonist (p.**54**), nicotine was mainly used and the stimulating effect of nicotine was compared with that of other cholinergic agonists and K^+. The effect of a range of cholinergic antagonists on agonist evoked release was determined in an attempt to classify the nicotinic heteroreceptor as either a C_6 or a C_{10} related nAChR.

4.2 METHODS

4.2.1 Effect of cholinergic agonists, veratridine and K^+ on the release of [^{3}H]DA from striatal synaptosomes

The efficacy of a range of nicotinic agonists, veratridine and K^+ (for chemical strutures see Appendix) in releasing[^{3}H]DA from perfused striatal synaptosomes was determined using a single pulse of each stimulus at t = 40 min.

ACh was dissolved in perfusion medium (in the absence of acetylcholinesterase inhibitors) immediately before use.

4.2.2 Repetitive stimulation

After the 40 min washout period the synaptosomes were stimulated 3 times at 30 min intervals (Fig. 3.14; p.**146**). The evoked release was calculated by summing the radioactivity released in each peak and subtracting the basal efflux (see p.**142**). The radioactivity released by the second and third stimulations (S_2 and S_3) was expressed as a percentage of the initial response to give S_2/S_1 and S_3/S_1 values respectively.

The effect of the DA uptake inhibitor nomifensine (50 µM) on the nicotine (1 µM) evoked release was determined by including nomifensine in the perfusion medium from the beginning of the washout period.

4.2.3 Ca^{2+}-dependency of stimulated [^{3}H]DA release

To study the Ca^{2+}-dependency of [^{3}H]DA release evoked by various stimuli, four perfusion systems were operated in parallel, two were continually washed with Ca^{2+}-free medium (p.**137**) and two were washed with normal medium. Pulses of stimulating agents pre-

pared in either Ca^{2+} -containing or Ca^{2+}-free medium were administered in the normal manner at t = 40, 70 and 100 min. The release in the Ca^{2+}-free medium was expressed as a percentage of the release obtained by an analogous pulse (of equivalent perfusion time) in normal medium.

4.2.4 Effect of cholinergic antagonists on stimulated [^{3}H]DA release

The effect of various cholinergic antagonists on K^+ (16 or 28 mM) and agonist (0.5 - 5 µM) evoked release was determined as described in Section 3.2.6b (p.**145**). Stimulating agents were prepared in normal and antagonist-containing medium.

4.2.5 The effect of pharmacological agents on synaptosomal integrity

To determine whether exposure of the striatal synaptosomes to various pharmacological agents caused any damage which could lead to leaky membranes, the occluded LDH activity was measured (Section 2.2.3, p.**67**) after incubation of a sample of synaptosomes for 2 min with each agent.

Statistical analysis of all data was performed using a two tailed Students' t-test. A value of $P < 0.1$ was considered to be statistically significant.

4.3 RESULTS

4.3.1 The effect of nicotine concentration on the release of [^{3}H]DA from striatal synaptosomes

The release of [^{3}H]DA from the perfused synaptosomes evoked by (-)-nicotine (free base) was studied over the concentration range 10^{-8} - 10^{-3} M. A plot of [^{3}H]DA released (above spontaneous)/mg protein, against log nicotine concentration showed that the release of radiolabelled transmitter by nicotine was dose-dependent, with the response saturating at concentrations greater than 0.1 mM (Fig. 4.1). Evoked release of [^{3}H]DA could be detected using nicotine concentrations as low as 10^{-8} M but the peak of radioactivity released by this concentration was small, making it difficult to quantify. A detailed study was carried out at higher concentrations (10^{-7} - 10^{-3} M) where the peaks of released tritium were well defined. Analysis of the dose-response data using the Hill plot (Fig. 4.2) gave an EC_{50} value of 3.8 μM.

The duration of the response, measured by counting the number of fractions contributing to the peak of stimulation before return to baseline, increased with increasing concentration of nicotine. At low concentrations (0.01 - 1 μM) the peak was spread over 4 fractions, at 0.5 - 10 μM nicotine over 4 - 6 fractions and at concentrations greater than 50 μM over 6-8 fractions (Table 4.1).

4.3.2 The release of [^{3}H]DA from striatal synaptosomes by cholinergic agonists

All the cholinergic agonists tested, stimulated the release of [^{3}H]DA from the perfused striatal synaptosomes (Table 4.1). The release of radiolabel evoked by nicotine, DMPP and cytisine was not

Fig. 4.1. The effect of nicotine concentration on the release of [^{3}H]DA

The release of [^{3}H]DA by (-)-nicotine (0.1 - 1000 μM) was measured by exposing the perfused striatal synaptosomes to pulses of (-)-nicotine at t = 40 (S_1). The radioactivity released in response to stimulation was expressed as fmoles [^{3}H]DA released above spontaneous level/mg protein. Results are expressed as the mean ± SEM (n > 4).

Fig. 4.1. Log dose-response curve for (-)-nicotine.

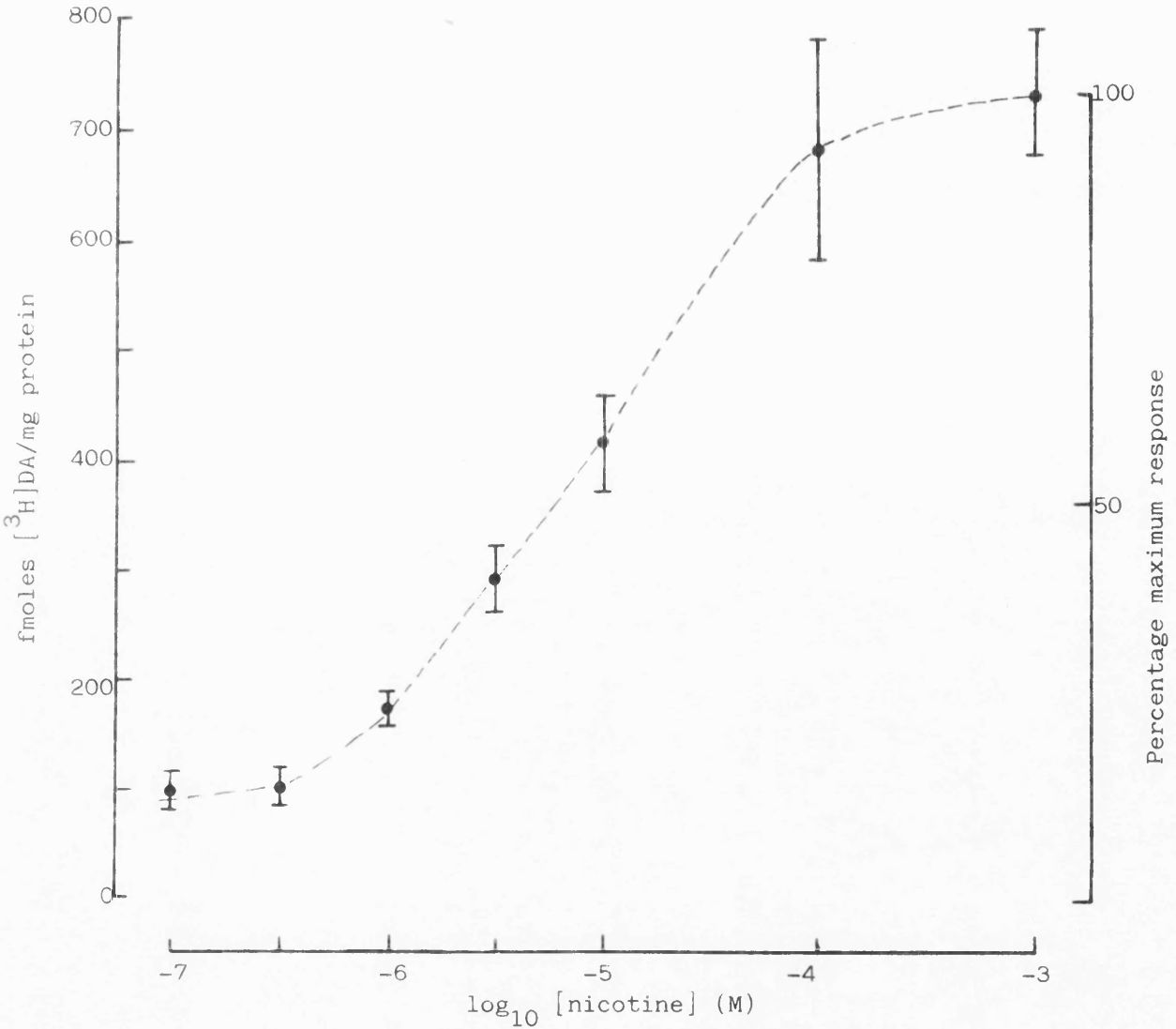


Fig. 4.2. Hill Plot of the dose-response data for (-)-nicotine.

The data obtained for the log dose response curve for (-)-nicotine (Fig. 4.1) were analysed using the method of Hill (1910). The logarithmic Hill equation is as follows:

$$\log \left(\frac{P}{100-P} \right) = n \log [L] - \log EC_{50}$$

where P is the response expressed as a percentage of the maximum response obtained at 1 mM nicotine, n is the interaction coefficient, EC_{50} is the concentration which produces half the maximum response, and L is the concentration of the ligand used (nicotine).

A plot of log $(\frac{P}{100-P})$ versus log [nicotine] gives a straight line, the slope being n and the intercept on the ordinate, -log EC_{50}.

An EC_{50} value of 3.8 μM was obtained and a Hill coefficient of 0.731, (r^2 = 0.959).

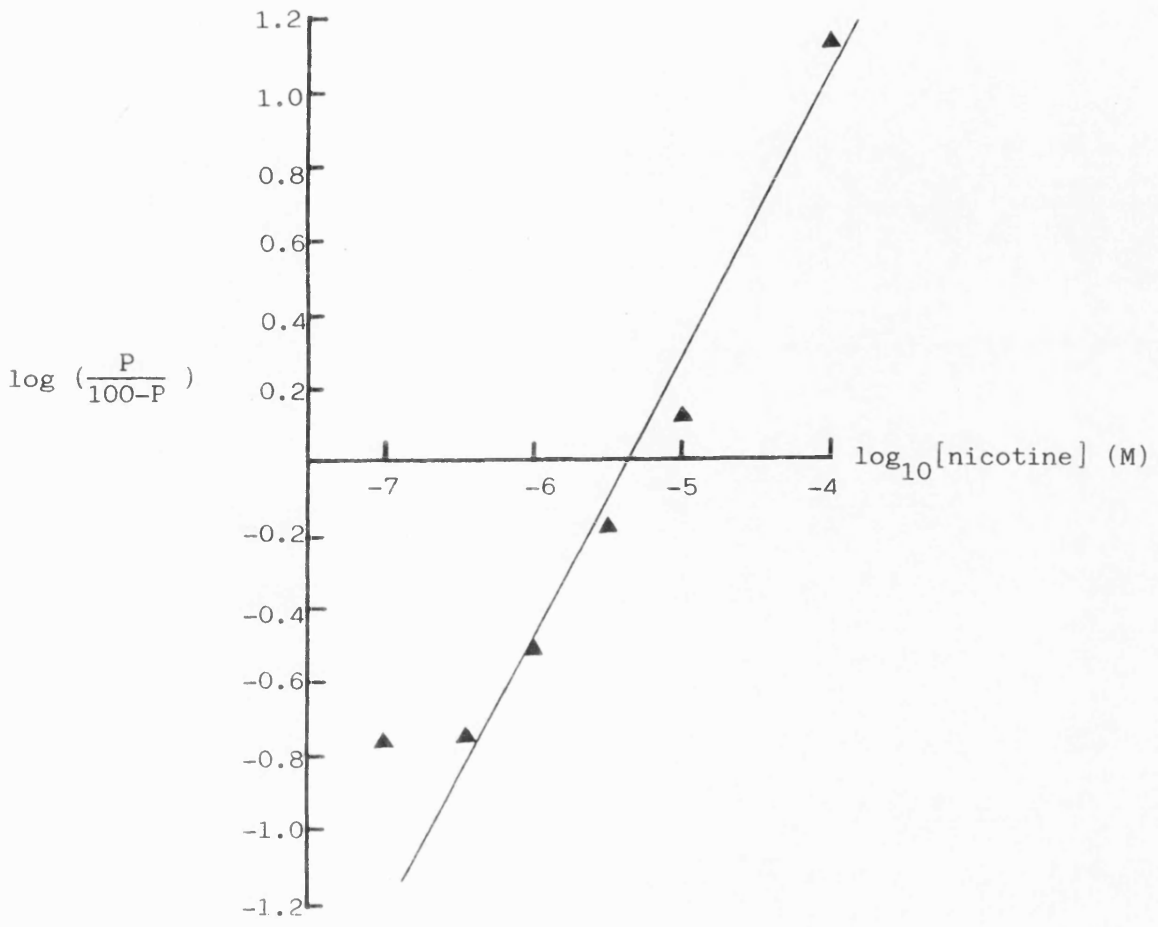


Fig. 4.2. Hill Plot of the (-)-nicotine dose-response curve data(Fig. 4.1).

Table 4.1. The effect of various stimuli on the release of [^{3}H]DA from striatal synaptosomes.

Concentration	Stimulus	Release (fmoles [^{3}H]DA/mg protein)	Duration of response: number of fractions
1 μM	(-)-nicotine	174.2 ± 9.8	4-6
	(-)-nicotine hydrogen (+)-tartrate	184.9 ± 13.7	4-6
	Cytisine	207.3 ± 22.6	4-5
	DMPP	153.4 ± 13.2	4-5
	Lobeline	133 (n=1)	4
	Coniine	102 (n=1)	4
16 mM	K^+	170.9 ± 13.0	3-4
additional 16 mM	Na^+	57.3 ± 9.7*	3-4
10 μM	(-)-nicotine	419 ± 85.9	4-6
	ACh	381 ± 10	3-5
	Choline	225 ± 26 (n=2)	4
	oxotremorine	176 ± 20 (n=2)	4
28 mM	K^+	411.5 ± 39.2	3-4
100 μM	(-)-nicotine	689.5 ± 96	6-8
	ACh	721 ± 183 (n=2)	4-5
	Choline	517 ± (n=1)	4
	Carbamylcholine	421 ± 52.3**	4-5
10 μM	Veratridine	1423 ± 291	14-16
30 μM	"	4568 ± 150	15-17
100 μM	"	12780 ± 635	15-17

The release of [^{3}H]DA by various cholinergic agonists, K^+ and veratridine was determined by measuring the [^{3}H]DA released by a single pulse (S_1) of each agent at t = 40 min. The radioactivity released above the spontaneous level was calculated as fmoles [^{3}H]DA/mg protein and the results averaged, mean ± SEM (n > 4) or mean ± range (n=2).

* indicates that the value is significantly lower than the release evoked by nicotine (1 μM) with $P < 0.0005$.

** indicates that the value is significantly different from the release evoked by nicotine (100 μM) with $P < 0.05$.

due to structural damage of the synaptosomal membranes as determined by measurement of the occluded LDH activity (Table 4.2). At a low agonist concentration (1 μM) the release by (-)-nicotine, (-)-nicotine hydrogen (+)-tartrate and the two ganglionic agonists DMPP and cytisine were comparable. However, the release evoked by the same concentration of the ganglionic agonists lobeline and coniine was slightly lower.

The effect of ACh and choline was determined at 10 and 100 μM. The release by ACh was similar to the release evoked by nicotine at the same concentration, whereas the [^{3}H]DA released by choline was substantially lower. The release of [^{3}H]DA evoked by carbamylcholine (100 μM) was comparable with that released by nicotine and ACh at 10 μM and was significantly lower ($P < 0.05$) than that obtained using nicotine or ACh at an equal concentration.

The muscarinic agonist oxotremorine (10 μM) evoked the release of [^{3}H]DA at a level equivalent to the [^{3}H]DA released by the nicotinic agonists at a lower concentration of 1 μM.

The time courses of the responses produced by each nicotinic agonist at 1 μM were comparable, whereas at high agonist concentrations (100 μM) the time course of the response to nicotine was notably longer than that of the other cholinergic agonists that were tested (Table 4.1).

4.3.3 The release of [^{3}H]DA by K^+ and veratridine

A 100 μl pulse of K^+(16 mM) released about the same amount of [^{3}H]DA as (-)-nicotine (1 μM); Table 4.1). In contrast, a sample containing elevated Na^+ (additional 16 mM) evoked only a small amount of [^{3}H]DA release.

Table 4.2. The effect of various pharmacological agents on striatal synaptosome integrity; determined by measurement of the occluded LDH activity after incubation of a sample of synaptosomes for 2 min with each agent.

Pharmacological agent	Final concentration	Occluded LDH activity, percentage of total activity
No addition	N/A	78.5 ± 1*
(-)-nicotine	20 μM	78
DMPP	"	79.5
Cytisine	"	77.5
Pempidine	10 μM	77.5
DHβE	10 μM	78.5
Hexamethonium	10 μM	79
K^+	16 mM	77.5
DMSO**	0.02% (v/v)	78

* Result expressed as mean ± S.D. (n=5)

All other values are the average of 2 determinations (variance ± 1%)

** Dimethylsulphoxide (used to dissolve NSTX).

Veratridine (10 - 100 μM) was very effective at eliciting release of [^{3}H]DA. This effect was not due to the presence of ethanol (used to dissolve the alkaloid), as perfusion with a pulse of medium containing an equivalent concentration of ethanol (0.25% v/v) that was present in a sample of veratridine (100 μM), caused no observable change in the basal release. The time course of the veratridine induced response was considerably longer than the responses to nicotine or K^+. At high concentrations of veratridine (30 - 100 μM) the duration of response was almost 40 min. In comparison, K^+ (16 and 28 mM) evoked release of short duration, the peak of radioactivity being spread over 3-4 fractions with a sharp increase and return to basal release (see also Fig. 3.11).

4.3.4 The Ca^{2+}-dependency of stimulated [^{3}H]DA release

The [^{3}H]DA released in response to the nicotinic agonists (-)-nicotine and DMPP, and the depolarizing agents K^+ and veratridine, was shown to be partially Ca^{2+}-dependent (Table 4.3). The response to (-)-nicotine and DMPP at a low concentration of 0.5 μM showed a greater requirement for Ca^{2+} than at the higher concentration of 1 μM. Similarly, a greater percentage of the response by veratridine (10 μM) was Ca^{2+}-dependent than at a higher concentration of 30 μM.

4.3.5 The effect of repetitive stimulation

The effect of repeated stimulation with (-)-nicotine (0.1 - 1000 μM) on the amount of [^{3}H]DA released is shown in Fig. 4.3. Over the concentration range 1 - 10 μM the [^{3}H]DA released by the second stimulation (S_2) was at least 80% that of S_1.

Table 4.3. The Ca^{2+}-dependency of stimulated [^{3}H]DA release

Stimulus	Concentration	Percentage normal response
(-)-nicotine	0.5 μM	29.5
(-)-nicotine	1 μM	40.6
DMPP	0.5 μM	36.3
DMPP	1 μM	54.7
K^+	28 mM	36.1
Veratridine	10 μM	58.7
Veratridine	30 μM	77.4

The Ca^{2+}-dependency of [^{3}H]DA release evoked by nicotinic agonists (nicotine and DMPP) and depolarizing agents (K^+, veratridine) was determined by comparing the release in the absence of Ca^{2+} with that in normal perfusion medium.

The synaptosomes in the test systems were perfused with Ca^{2+}-free medium from the beginning of the washout period until the end of the experiment.

Results expressed as the mean percentage of the control response (in normal perfusion medium). Variance ± 10%, n > 4.

The possibility that reuptake of [^{3}H]DA may occur, and hence affect the levels of [^{3}H]DA released, was examined by the inclusion of the DA uptake inhibitor nomifensine (50 μM) in the perfusion medium. This drug had no effect on the percentage S_2/S_1 and S_3/S_1 values obtained for (-)-nicotine (1 μM) suggesting that little reuptake of the released [^{3}H]DA occurred.

At concentrations less than 1 μM and greater than 10 μM the release by S_2 was lower than S_1. Comparison of the radioactivity evoked by the third pulse (S_3) with S_1 was only made over the range 1 - 1000 μM. At 1 μM nicotine, release by S_3 was comparable with that of S_2 and S_1, but at concentrations greater than 1 μM the S_3 response was reduced.

The S_2/S_1 and S_3/S_1 values were obtained for other nicotinic agonists (Table 4.4). At a concentration of 1 μM all the nicotinic agonists studied showed little attenuation of the responses on repetitive stimulation; S_2/S_1 and S_3/S_1 values were similar to those of (-)-nicotine. However, K^+ (16 mM), which evoked approximately the same amount of [^{3}H]DA release as 1 μM concentrations of the agonists (Table 4.1), gave a S_3/S_1 value significantly lower ($P < 0.01$) than the S_3/S_1 values for the nicotinic agonists (1 μM).

Included in Table 4.4 are the results obtained using high concentrations (100 μM) of cholinergic agonists. (-)-Nicotine, ACh and carbamylcholine repeatedly stimulated the release of [^{3}H]DA resulting in comparable S_2/S_1 values, although the S_2/S_1 value for carbamylcholine was slightly higher ($P < 0.1$) than that for nicotine.

Fig. 4.3. Repetitive stimulation of striatal synaptosomes with (-)-nicotine (0.1 - 1000 μM).

Striatal synaptosomes preloaded with [^{3}H]DA were repeatedly stimulated with (-)-nicotine at t = 40,70,100 min. The release of [^{3}H]DA evoked by the second (S_2) and third (S_3) responses were expressed as a percentage of the initial response (S_1) to give S_2/S_1 and S_3/S_1 values.

Results expressed as mean ± SEM (n > 4).

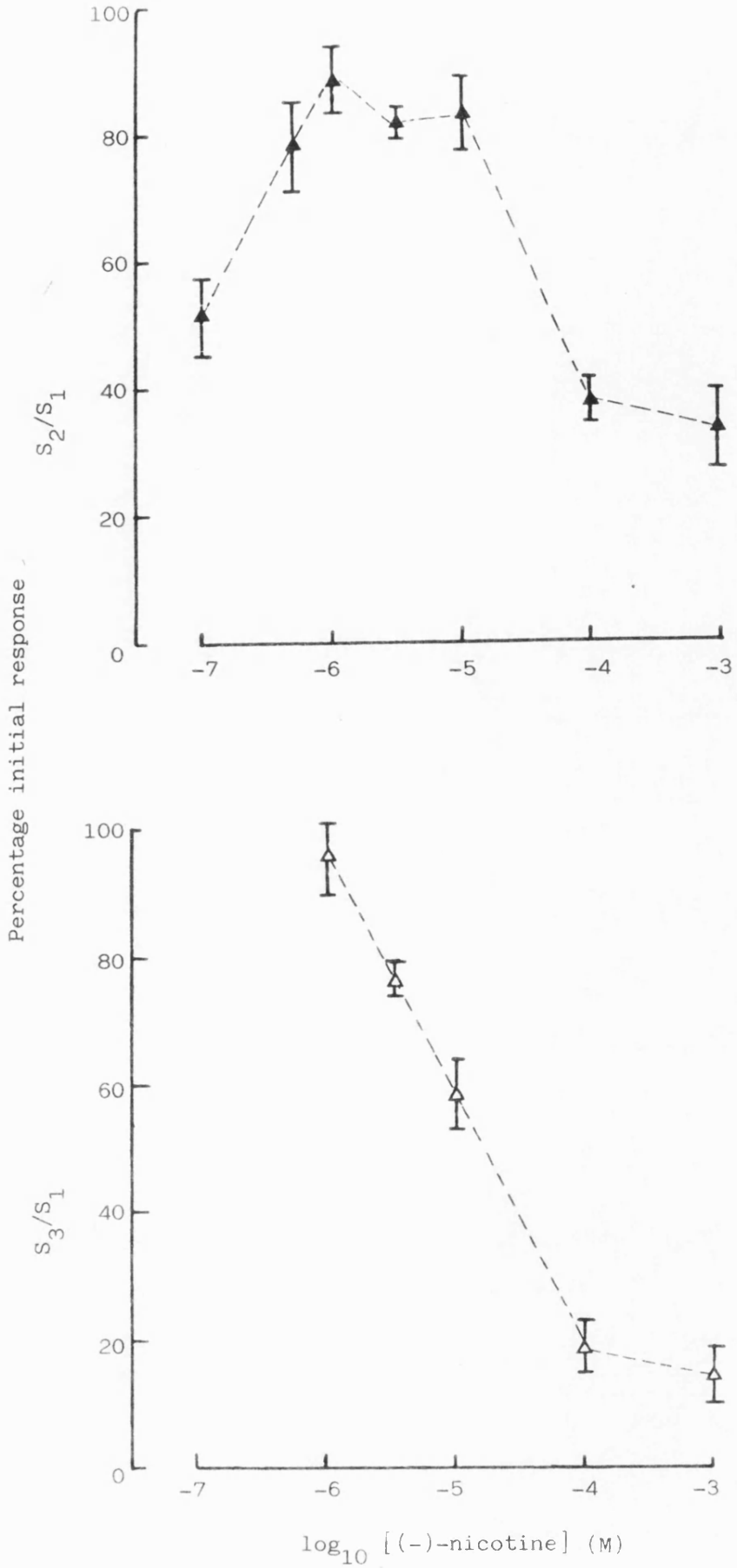

Percentage initial response
S_2/S_1
S_3/S_1
100
80
60
40
20
0
-7
-6
-5
-4
-3
$\log_{10}$ [(-)-nicotine] (M)

Fig. 4.3

Table 4.4. Repetitive stimulation

Stimulus	Concentration	S_2/S_1 (%)	S_3/S_1 (%)
(-)-nicotine	1 μM	89.6 ± 5.4	96.2 ± 4.9
(-)-nicotine + nomifensine (50 μM)	1 μM	89.5 ± 3.5 (n=2)	101.5 ± 11.5(n=2)
(-)-nicotine hydrogen (+)-tartrate	1 μM	90.1 ± 3.5	95.5 ± 3.5
DMPP	1 μM	110.3 ± 7.2*	104.2 ± 11.9
Cytisine	1 μM	93.9 ± 5.5	84.4 ± 6.5
K^+	16 mM	90.8 ± 5.5	67.8 ± 5.7**
K^+	28 mM	91.2 ± 4.6	ND
(-)-nicotine	100 μM	38 ± 2.9	19.5 ± 2.9
ACh	100 μM	46 ± 2.5(n=2)	ND
Carbamylcholine	100 μM	49.7 ± 5.1***	ND

Striatal synaptosomes were repeatedly stimulated at 30 min intervals (see Fig. 3.14; p.**146**) with a pulse of the same stimulus. The [^{3}H]DA released by the second (S_2) and third (S_3) pulses were expressed as a percentage of the first stimulation (S_1).

Results expressed as mean ± SEM, n > 4. or mean ± range, n = 2.

* Denotes the response is significantly different from the (-)-nicotine (1 μM) S_2/S_1 value with $P < 0.1$
** Denotes the response is significantly lower than the (-)-nicotine (1 μM) S_3/S_1 response with $P < 0.01$
*** Denotes the response is significantly different from the (-)-nicotine (100 μM) S_2/S_1 value with $P < 0.1$.

Fig. 4.4. The effect of (+) and (-)-nicotine on the release of [^{3}H]DA from perfused striatal synaptosomes.

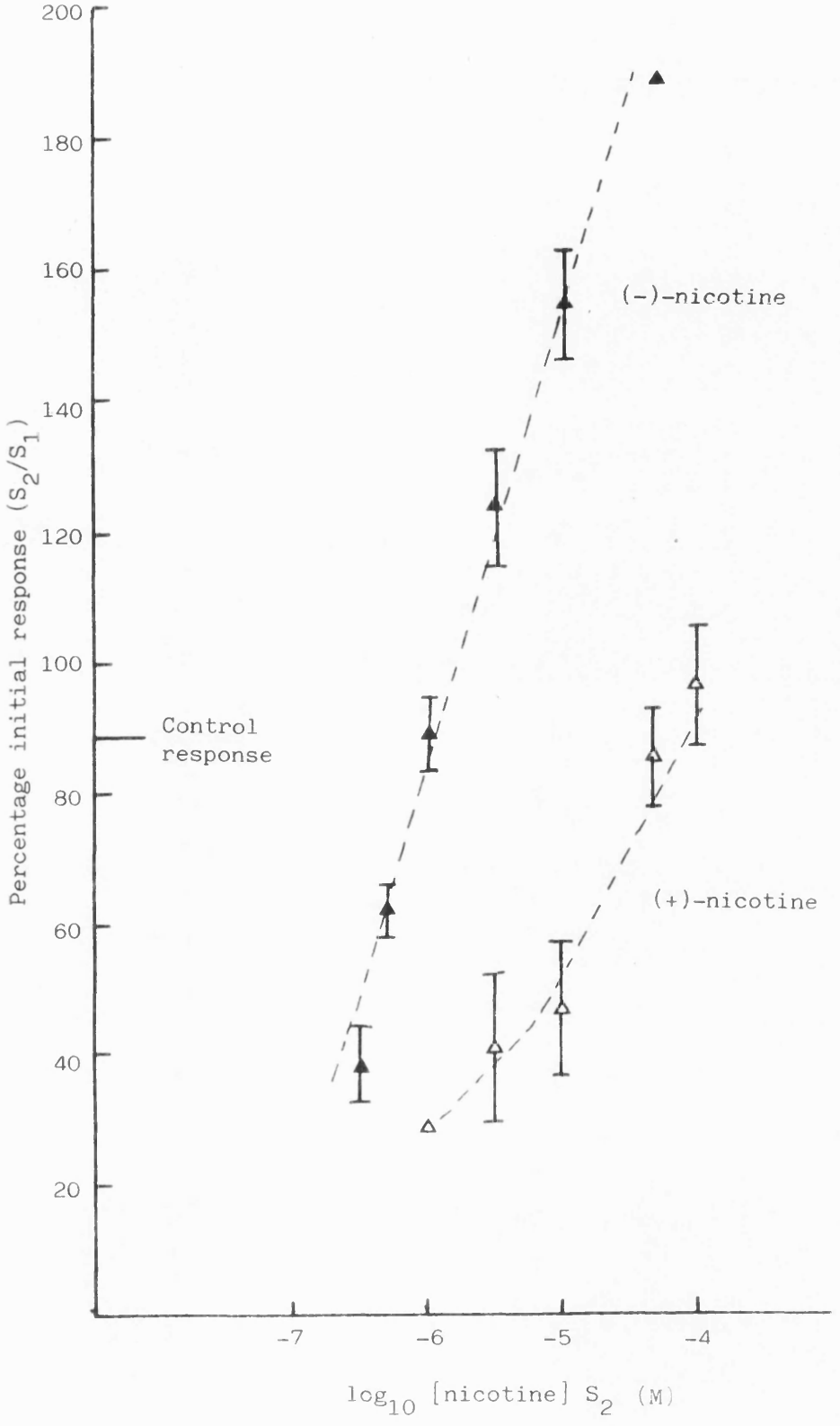
Striatal synaptosomes were stimulated with 2 pulses of nicotine at t = 40 and t = 70 min.

S_1 = (-)-nicotine hydrogen (+)-tartrate (1 μM)

S_2 = (+)-nicotine di(-)-tartrate or (-)-nicotine hydrogen (+)-tartrate (0.3 - 100 μM).

The x-axis indicates the log concentration of nicotine in S_2 and the y-axis shows the response by S_2 as a percentage of the S_1 response, (S_2/S_1 value).

The S_2/S_1 value obtained from 2 successive stimulations of 1 μM (-)-nicotine hydrogen (+)-tartrate was used as the the control response.


200
180
160
140
120
100
80
60
40
20
Percentage initial response (S_2/S_1)
Control response
(-)-nicotine
(+)-nicotine
-7
-6
-5
-4
$\log_{10}$ [nicotine] S_2 (M)

The mean S_2/S_1 and S_3/S_1 values obtained for each stimulus (Table 4.4) were used as control values in the experiments carried out to determine the effect of nicotinic antagonists on evoked [^{3}H]DA release from striatal synaptosomes.

4.3.6 Stereoselectivity of the nicotine response

The sample of (+)-nicotine obtained for these studies was in the form (+)-nicotine di(-)-tartrate and was >99% pure. To compare its efficacy with its enantiomer, a tartrate salt of the (-)-form was used, (-)-nicotine hydrogen-(+)-tartrate, the (-)-nicotine tartrate being of equal potency to the free base (Table 4.1).

By initially stimulating with a pulse of (-)-nicotine (1 μM) followed by a pulse of either (+) or (-)-nicotine (0.3 - 100 μM) the stereoselectivity of the response was clearly demonstrated (Fig. 4.4). The dose-response curve for the (+) enantiomer was markedly shifted to the right. Taking the S_2/S_1 value for the standard condition of successive pulses of 1 μM (-)-nicotine as the control response an equivalent S_2/S_1 value was only obtained when the concentration of (+)-nicotine was increased to 100 μM. This corresponds to a relative potency of (-) : (+) of 100 : 1.

4.3.7 The effect of cholinergic antagonists on [^{3}H]DA release from perfused striatal synaptosomes

The effect of a single concentration nicotinic antagonists on the release of [^{3}H]DA stimulated by low concentrations (< 5 μM) of nicotinic agonists ((-)-nicotine, DMPP and cytisine) is

Table 4.5. The effect of nicotinic antagonists on stimulated [^{3}H]DA release from striatal synaptosomes.

Antagonist	(-)-nicotine		DMPP			Cytisine	K^+
	0.5 μM	1 μM	0.5 μM	1 μM	5μM	1 μM	16 and 28 mM
DHβE 0.5 μM	ND	27.1	ND	32.4	ND	42.1	83.9
NSTX 0.05 μM	62	46.5*	ND	ND	59.2	ND	106
Pempidine 5 μM	27.4	37.8	51.5	61.3	ND	ND	95.8
Mecamylamine 5 μM	55.5*	45.3	68*	54.4	ND	49.4	98.4
αBGT 0.25 μM	87	98.5	97.5*	112.2	ND	ND	95.3
D-tubocurarine 10 μM	ND	ND	ND	85	ND	ND	ND
Hexamethonium 10 μM	ND	87	ND	ND	ND	100.5	ND
Decamethonium 10 μM	ND	66.7	ND	ND	ND	ND	ND
Chlorisondamine 5 μM	ND	84*	ND	84*	ND	82*	80*

Perfusion was carried out as described in Fig. 3.14. The mean S_2/S_1 value in the presence of an antagonist was calculated and expressed as a percentage of the S_2/S_1 value obtained in normal medium (Table 4.4). Results are from at least 4 experiments with variance <10% except those indicated by * which denotes a result from a single experiment.

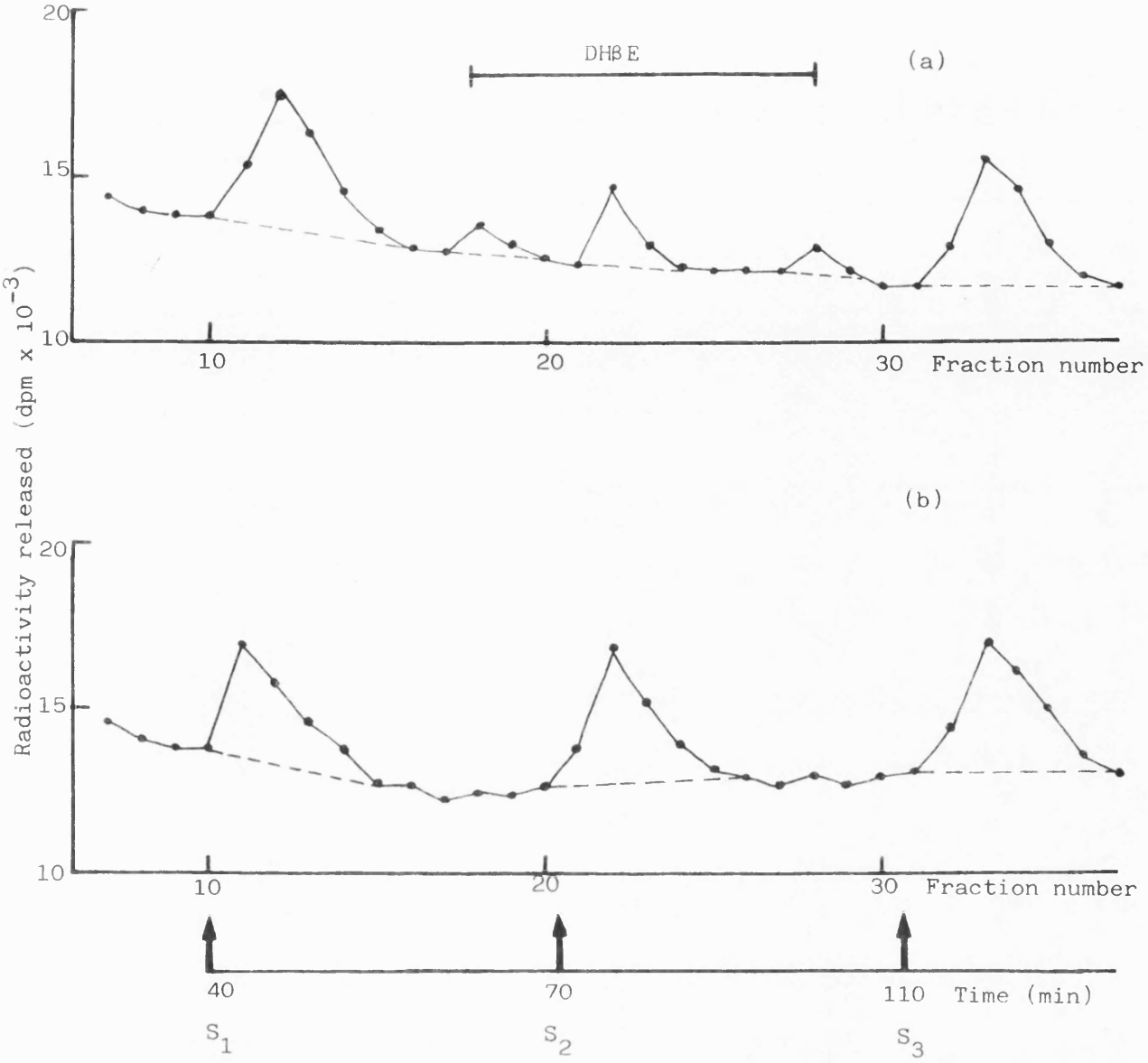


Fig. 4.5. The effect of DHβE (0.5 μM) on DMPP (1 μM) evoked release of [^{3}H]DA from striatal synaptosomes.

Striatal synaptosomes (200 μl, 0.54 mg protein/filter) were perfused and stimulated at the times indicated by the arrows with a pulse of DMPP (1 μM).

a) Test system, DHβE (0.5 μM) was present in the perfusion medium for the duration of the bar, the small peaks either side of S_2 correspond to changes in the perfusion medium.

b) Control system, continual perfusion with normal perfusion medium.

summarised in Table 4.5. The concentration of antagonist was selected on the basis of reports in the literature. Using the protocol devised in Fig. 3.14 antagonism of agonist-evoked release was clearly demonstrable by comparing the S_2 responses in the presence and absence of antagonist. This is illustrated in Fig. 4.5 which shows the effect of dihydro-β-erythroidine on DMPP-evoked [^{3}H] DA release. The possibility that some antagonists might cause non-specific transmitter release (thereby obscuring any antagonism of the nicotinic response) by disruption of the synaptosomal membrane was tested by measurement of the occluded LDH activity. None of the antagonists tested appeared to cause any such effects (Table 4.2).

a) Neosurugatoxin (NSTX)

Studies with NSTX were carried out during the development of the perfusion system when slightly higher concentrations of agonists were used and the antagonist was present from the beginning of the washout period; the agonist-evoked release in the presence of the antagonist was compared with a parallel control system (Fig. 4.6). Using this method, a freshly prepared solution of NSTX (0.05 μM) was shown to be an effective inhibitor of DMPP (5 μM) evoked release of [^{3}H]DA, inhibiting the normal response by about 80%. However, there was only a small amount of starting material which was stored as a stock solution as described in "Materials" and because NSTX is unstable in aqueous solution (Kosuge *et al.*, 1982) a deterioration of the activity of NSTX was observed (Table 4.6). After 57 days in solution NSTX was ineffective at preventing DMPP (5 μM) evoked transmitter release.

Table 4.6. Deterioration of NSTX activity

Number of days in solution	Percentage inhibition of normal response (mean ± S.D., n > 3)
1	87.5 ± 10.6
5	52.3 ± 8.1
11	45.0 ± 7.2
50	17.2 ± 9.3
57	0

The deterioration of NSTX activity was determined by measuring the inhibition by NSTX (0.05 μM) of the normal DMPP (5 μM) evoked release of [^{3}H]DA from striatal synaptosomes.

Fig. 4.6. The effect of neosurugatoxin on stimulated release of [^{3}H]DA from striatal synaptosomes.

Striatal synaptosomes (0.75 mg protein/filter), preloaded with [^{3}H]DA (0.1 µM, 15 Ci/mmol) were perfused with a) normal medium or b) medium containing 5 x 10^{-8}M NSTX for 110 min (////). In test systems (a and b) 100 µl pulses of (DMPP 5 µM; ↓) or K^+ (28 mM; ⇓), resulted in responses indicated by the arrows.

In a control system(c, not shown) release throughout the perfusion period was measured and this was subtracted from the transmitter release profiles of the test systems (a and b).

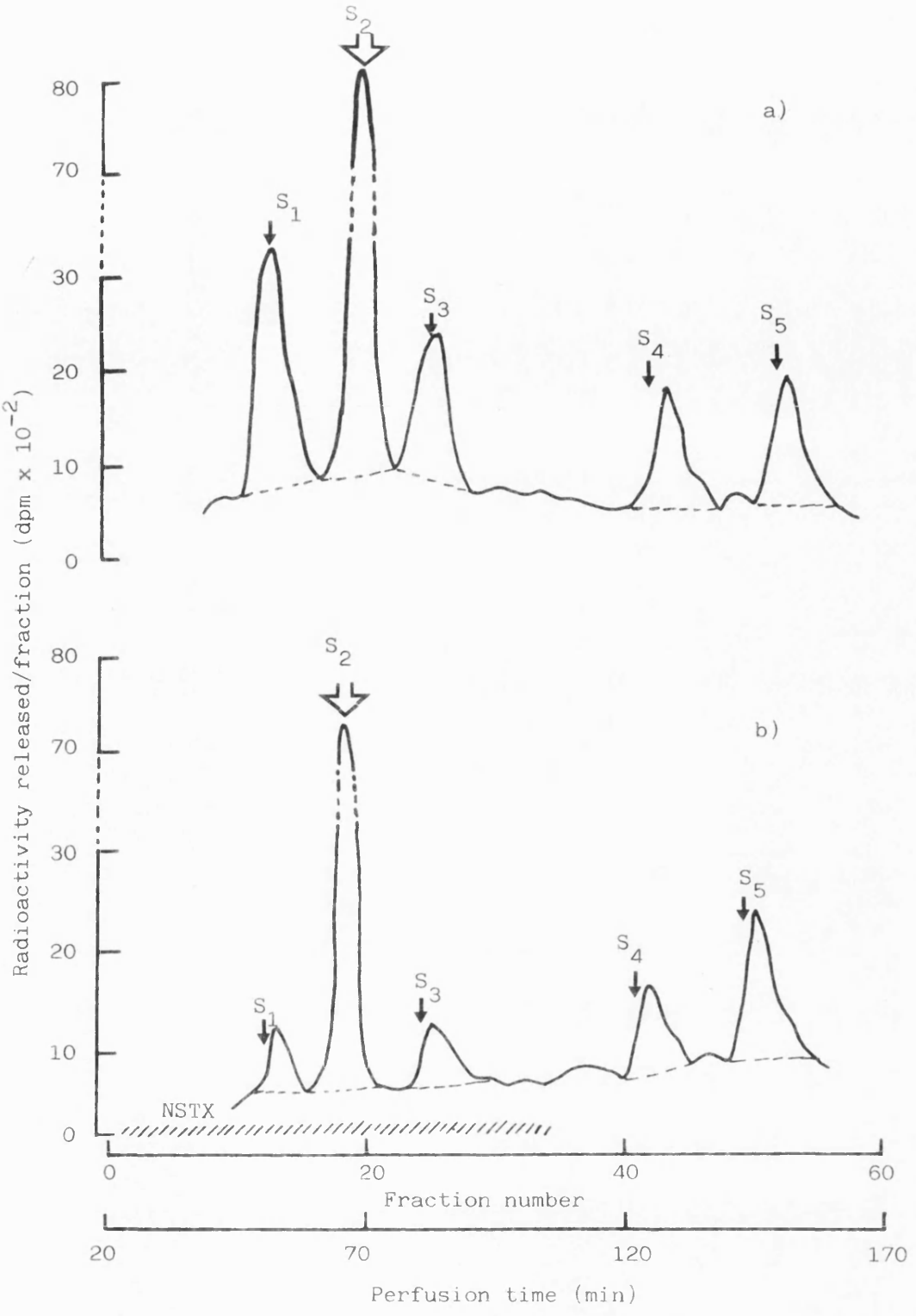


Fig. 4.6 The effect of neosurugatoxin on stimulated release of [^{3}H]DA from striatal synaptosomes

The results shown in Table 4.5 of the effect of NSTX (0.05 μM) on DMPP (5 μM) and nicotine (0.5 - 1 μM) evoked release are the average of a series of experiments carried out during the period when the toxin was active. The observed antagonism by NSTX is therefore probably an underestimate of its true potency which was observed on day 1 (Table 4.6). The effect of NSTX on nicotine (0.5 - 1 μM) stimulated [^{3}H]DA release was carried out using toxin which had been in solution for 20 - 30 days. The observed antagonism of about 50% further demonstrated the potency of NSTX. The specificity of the action of NSTX was indicated by its lack of effect on K^+ evoked release (Table 4.5). The effect of NSTX was also shown to be reversible (Fig. 4.6); the response to DMPP (5 μM) gradually recovered after withdrawal of the toxin and washing with normal perfusion medium. After 20 min, the response (S_4) had recovered to 83% control and after 40 min the response (S_5) was 100% of the control value.

b) α-Bungarotoxin (α-BGT)

Using the normal perfusion schedule (Fig. 3.14) α-BGT (0.05 - 0.25 μM) was ineffective at inhibiting agonist evoked release of [^{3}H]DA from striatal synaptosomes and did not affect the release by K^+ (Table 4.5). However, with prolonged exposure to α-BGT (0.125 μM) a partial antagonism of (-)-nicotine (10 μM) evoked release was observed (Table 4.7). After 30 min perfusion with the neuromuscular blocking agent a 10% block was observed, and after 55 min the normal response was blocked by 30%.

Table 4.7. The effect of α-BGT (0.125 μM) on (-)-nicotine (10 μM) stimulated release of [^{3}H]DA from striatal synaptosomes

Perfusion details	S_2/S_1 (%)	S_3/S_1 (%)
normal (control)	99.5 ± 0.5	74 ± 5
+ α-BGT (0.125 μM)	89.5 ± 2.5	50.5 ± 5.5

Striatal synaptosomes preloaded with [^{3}H]DA were perfused and stimulated 3 times with nicotine (10 μM) at t = 40, 95, 120 min. In the test system at t = 65, α-BGT (0.125 μM) was introduced into the perfusion medium and continued until the end of the experiment. The release of radioactivity (above the spontaneous level) by the second and third stimulations (S_2 and S_3) were expressed as a percentage of the initial response (S_1).

Results expressed as mean ± range (n = 2).

c) Perhydrohistrionicotoxin The effect of H_{12}HTX on stimulated ((-)-nicotine and K^+) release of [^{3}H]DA from striatal synaptosomes was studied by repeatedly stimulating at 40 min intervals (instead of 30 min). This was to allow a 15 min pre-exposure of the synaptosomes to H_{12}HTX before S_2 (after Kato and Changeux, 1976), by introducing the toxin into the medium at t = 65 min. In some experiments, the toxin was present throughout the remainder of the perfusion, in others it was present only for 30 or 35 min.

In the control systems repetitive stimulation with pulses of (-)-nicotine (1 or 10 μM) or K^+ (16 mM) at 40 min intervals resulted in S_2/S_1 and S_3/S_1 values (Table 4.8) which were within the ranges obtained using stimulation at 30 min intervals (see Fig. 4.3 and Table 4.4).

Table 4.8. Repetitive stimulation of striatal synaptosomes at 40 min intervals.

Stimulus	S_2/S_1 (%)	S_3/S_2 (%)
(-)-nicotine 1 μM	86.5 ± 5.1 (5)	94 ± 2.6 (4)
(-)-nicotine 10 μM	84 ± 6.8 (4)	57.6 ± 3.2 (4)
K^+ (16 mM)	86 ± 5 (2)	65.5 ± 2.5 (2)

Results expressed as the mean of n determinations.
For $n \geq 4$, the variance is expressed as the SEM and for n = 2 as the range.

The effect of increasing concentrations of H_{12}HTX on 1 μM nicotine evoked striatal [^{3}H]DA release was examined (Fig. 4.7). A steep dose-response curve over a narrow range of toxin concen-

tration (1 - 5 μM) was observed and at the highest concentration used (5 μM) the normal response was inhibited by approximately 50%. Using identical experimental conditions, K^+ (16 mM) evoked release was unaffected by the presence of 5 μM H_{12}HTX (Table 4.9). At a higher agonist concentration of 10 μM, antagonism by 5 μM H_{12}HTX was also observed, the normal response being reduced by 34%.

The effect of prolonged exposure to H_{12}HTX and the recovery from the inhibition was investigated (Table 4.9). When striatal synaptomes were stimulated a third time with 1 μM nicotine in the continued presence of 5 μM H_{12}HTX no further inhibition of the response by H_{12}HTX was observed. The S_3/S_1 value of 42.4 agrees with the S_2/S_1 value. Furthermore, if perfusion with the toxin was terminated 10 min before S_3 and washing was continued with normal perfusion medium the inhibition of the normal response was still observed. However, extending the wash period before S_3 to 15 min revealed that the effect of H_{12}HTX was reversible; for nicotine (10 μM) stimulated [^{3}H]DA release the S_3/S_1 value was about 90% of the control S_3/S_1 value.

d) Ketamine

The anaesthetic ketamine was also shown to be an effective inhibitor of (-)-nicotine (1 μM) stimulated release of [^{3}H]DA from striatal synaptosomes. At a concentration of 1 μM, ketamine inhibited approximately 40% of the normal nicotinic response whereas K^+ evoked release was unaffected (Table 4.9). However, higher concentrations of ketamine (10 μM) failed to inhibit nicotine evoked release. The inhibition produced by 1 μM ketamine was reversible, the S_3 response returned to about 80% control after a 10 min wash with normal perfusion medium.

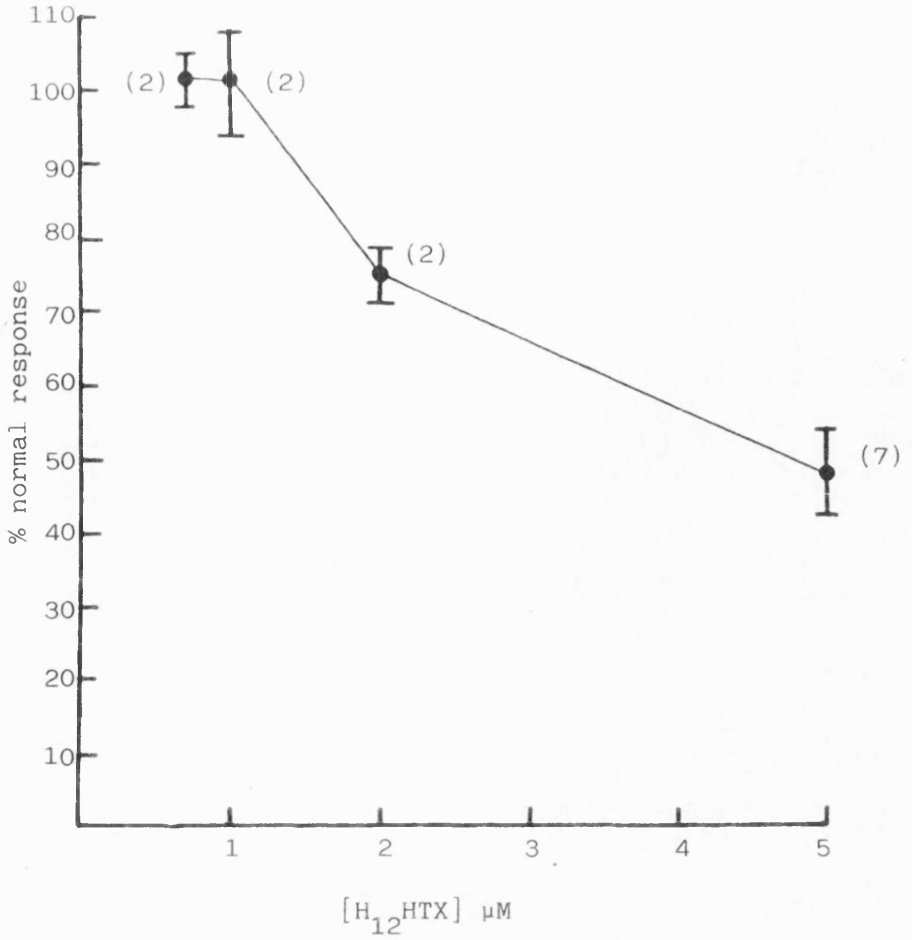


Fig. 4.7. Inhibition of nicotine (1 µM) evoked release of [^{3}H]dopamine from striatal synaptosomes by H_{12}HTX. Striatal synaptosomes were repeatedly stimulated at 40 min intervals with (-)-nicotine (1 µM), initially in the absence and then either in the absence or presence of H_{12}HTX. The S_2/S_1 percentage obtained in the presence of the toxin was expressed as a percentage of the control S_2/S_1 value.

Results represent the mean of n determinations +/- the range for n = 2, and ± SEM n = 7.

Table 4.9. The effect of H_{12}HTX and ketamine on stimulated release of [^{3}H]DA from striatal synaptosomes.

1) The effect of H_{12}HTX (5 μM)

Perfused synaptosomes were stimulated 3 times at 40 min intervals and H_{12}HTX introduced into the perfusion medium 15 min before S_2 and continued throughout the S_3 response or stopped 10 or 15 min before S_3.

2) The effect of ketamine (1 and 10 μM)

Striatal synaptosomes were stimulated 3 times at 30 min intervals; ketamine was introduced 10 min before S_2 and continued for 30 min before return to normal perfusion medium.

The S_2/S_1 and S_3/S_1 values were calculated for the test systems and the results expressed as the percentage of analogous S_2/S_1 values obtained in control systems (Table 4.8).

Results are expressed as the mean of (n) determinations with a variance of <10%.

Table 4.9

Perfusion conditions	Percentage normal response			
	S_2/S_1	S_3/S_1 (in presence of channel blocker)	S_3/S_1 (after 10 min wash)	S_3/S_1 (after 15 min wash)
Striatal synaptosomes				
(-)-nicotine (1 μM) + H_{12}HTX (5 μM)	47.9 (7)	42.4 (4)	35.9 (2)	N.D.
(-)-nicotine (1 μM) + ketamine (1 μM)	63.7 (4)	N.D.	81.4 (4)	N.D.
(-)-nicotine (1 μM) + ketamine (10 μM)	97.1 (4)	N.D.	94.4 (4)	N.D.
(-)-nicotine (10 μM) + H_{12}HTX (5 μM)	66 (2)	N.D.	N.D.	88 (2)
K^+ (16 mM) + H_{12}HTX (5 μM)	91.9 (2)	N.D.	N.D.	92.5 (2)
K^+ (16 mM) + ketamine (1 μM)	106 (2)	N.D.	94 (2)	N.D.

e) Other antagonists

Dihydro-β-erythroidine (DHβE, 0.5 μM) was an effective inhibitor of [^{3}H]DA release evoked by the three nicotinic agonists, nicotine, DMPP and cytisine (Table 4.5). Another two antagonists pempidine and mecamylamine, at a higher concentration of 5 μM were also potent inhibitors of agonist evoked release. The effect of pempidine was determined using continual exposure to the antagonist. For comparison, the effect of mecamylamine was studied by both continuous exposure and using the final perfusion schedule with the antagonist present during S_2 only. Identical results were obtained using the two different methods, suggesting that a 10 min pre-exposure to mecamylamine was sufficient for antagonism of the nicotinic response. The specificity of these antagonists was shown by the lack of inhibition of K^+-evoked release.

Preliminary studies were also carried out using D-tubocurarine (10 μM), decamethonium (10 μM), hexamethonium (10 μM) and chlorisondamine (5 μM). The only antagonist in this group to show notable inhibition of agonist evoked release at the concentration tested was the neuromuscular blocking agent decamethonium (Table 4.5).

To assess the reversibility of antagonism, S_3/S_1 values were determined after the removal of the antagonist and a 10 min wash with normal perfusion medium. These experiments clearly showed that there was full recovery after perfusion with DHβE (0.5 μM) but partial antagonism remained when mecamylamine (5 μM) or decamethonium (10 μM) had been present (Table 4.10).

Preliminary experiments were also carried out using DHβE (0.5 μM) and the muscarinic antagonist atropine (0.5 μM) to assess the nature of the receptor(s) mediating the [^{3}H]DA release evoked

by ACh (100 μM) and carbamylcholine (100 μM). Both agonists were sensitive to DHβE (0.5 μM) but antagonism by atropine (0.5 μM) was not observed (Table 4.11).

Table 4.10. Recovery of agonist stimulated release of [^{3}H]DA from striatal synaptosomes after exposure to various antagonists.

Antagonist	AGONIST (-)-Nicotine (1 μM)	Cytisine (1 μM)	DMPP (1 μM)
DHβE (0.5 μM)	99.8	94.5	90.2
Mecamylamine (5 μM)	78.6	79	83.6
D-tubocurarine (10 μM)	ND	ND	89.4
Hexamethonium (10 μM)	ND	93.5	ND
Decamethonium (10 μM)	56.1	ND	ND

Perfusion was carried out as described in Fig. 3.14 and the S_3/S_1 values calculated. The mean S_3/S_1 value obtained for each agonist (after exposure to an antagonist) was expressed as a percentage of the normal S_3/S_1 value (continual perfusion with normal medium; see Table 4.4).

Results are from at least 4 experiments, with variance <10%.

Table 4.11. The effect of atropine and DHβE on ACh and carbamylcholine stimulated release.

Antagonist	AGONIST	
	ACh (100 μM)	Carbamylcholine (100 μM)
Atropine (0.5 μM)	96	100
DHβE (0.5 μM)	73	26

Perfusion was carried out as described in Fig. 3.14.

The S_2/S_1 value in the presence of an antagonist was expressed as a percentage of the normal S_2/S_1 value for each agonist (Table 4.4).

Results are single determinations.

4.4 DISCUSSION

4.4.1 The release of [^{3}H]DA by a range of pharmacological agents

In this study a wide range of stimulating agents was shown to evoke [^{3}H]DA from striatal synaptosomes. The effect was not a result of synaptosomal damage leading to leakage of the radiolabel, as incubation of synaptosomes with each drug for 2 min had no effect on the level of the intrasynaptosomal enzyme marker, LDH (Table 4.2). Connelly and Littleton (1983) have also shown, by the measurement of LDH activity, that nicotine (100 μM) causes no structural damage to synaptosomes from whole brain. Additional information about the integrity of perfused synaptosomes was obtained by the analysis of the basal release. After stimulation with a pulse of pharmacological agent a return to basal release suggested that the synaptosomes were still intact.

The duration of each response was clearly dependent upon the concentration and nature of the stimulus (Table 4.1). Release evoked by both K^+ and nicotine was of short duration compared with the length of recovery from veratridine. However, the K^+ and nicotine responses were different (see Fig. 3.11), the peak of released radioactivity resulting from nicotine stimulation being slightly longer than K^+-evoked release. This observation is in agreement with previous reports (Arqueros *et al.*, 1978; Westfall, 1974a, b; Marien *et al.*, 1983). The nicotine-evoked release is probably an immediate response because even at high concentrations (100 μM), the duration of the nicotine response observed using the perfusion system described in this thesis was about the same as the distribution of a pulse of radioactivity (Sections 3.3.1 and 3.3.2). The 30 min interval between nicotine and K^+ stimulations is

therefore adequate.

In contrast, the long duration of veratridine evoked release may be a result of the action of veratridine on the inner surface of the voltage sensitive Na^+ channel, holding the channel open (Richie, 1979). A period of washing is therefore required to remove veratridine.

The effect of introducing an antagonist often resulted in a transient increase in the basal release (see Fig. 4.5). This small peak was spread over 1 - 2 fractions and at the most represented 15% of the initial response to 1 μM-agonist. A similar small peak was also observed at t = 90 min when perfusion with the antagonist was terminated and washing was continued with normal medium, suggesting that the effect was artefactual. However, the small peaks were partially dependent upon the antagonist. Decamethonium (10 μM) gave the most pronounced change in baseline and this might reflect its depolarising action.There are also reports in the literature that certain cholinergic antagonists show agonist properties e.g. the excitation of central neurons by curare-like antagonists (Krnjević, 1975) and the release of [^{3}H]DA from striatal tissue by high concentrations of mecamylamine (Giorguieff *et al.*, 1976; Mills and Wonnacott, 1984). Giorguieff and coworkers suggest that drugs may show agonist or antagonist properties according to the drug concentration and the tissue system employed.

4.4.2 Nicotine-evoked [^{3}H]DA release from striatal synaptosomes

The dose-dependent release of [^{3}H]DA from striatal synaptosomes by nicotine is in agreement with the results of Rowell and Winkler (1984) for the nicotine-evoked release of [^{3}H]ACh from

cortical synaptosomes. Nicotine concentrations greater than 1 mM were not tested because of the reported desensitisation of peripheral nAChRs to high and/or prolonged exposure to agonist (Katz and Thesleff, 1957) and the non-specific effects of nicotine at high concentrations (Arqueros *et al.* 1978; Marien *et al.*, 1983).

The nicotine-evoked release of [^{3}H]DA from striatal synaptosomes appeared linear over the concentration range 1 - 100 µM (Fig. 4.1) and the EC_{50} value obtained (Fig. 4.2) of 3.79 µM accords with the action of nicotine on the release of [^{3}H]DA from the amygdala (Rowell, 1987) and the nicotine stimulated release of catecholamines from bovine adrenal chromaffin cells (Adams *et al.*, 1986). There is no report of a full dose-response curve for nicotine facilitating [^{3}H]DA from striatal tissue, although Giorguieff-Chesselet *et al.* (1979) used nicotine concentrations as low as 1 µM to stimulate [^{3}H]DA release from striatal slices.

More often, concentrations of nicotine greater than 0.5 mM have been used to study the nicotinic heteroreceptor modulating DA release in the striata (Arqueros *et al.*, 1978; Westfall, 1974a; 1983a; Sakurai *et al.*, 1982; Marien *et al.*, 1983; Connelly and Littleton, 1983). The reason for using such high concentrations is not stated, but it is possible that the sensitivity of the systems employed for measuring the release were too low.

At high nicotine concentration ($>$ 100 µM) the observed reduction in the amount of [^{3}H]DA released by successive pulses may be a result of either depletion of the radiolabelled transmitter pools and/or desensitisation of the nicotinic receptors. Using the the revised perfusion system described in this thesis, the release of [^{3}H]DA by nicotine was therefore studied using more physiolo-

gically relevant concentrations. The nicotine concentration range chosen (1 - 10 μM) is consistent with the EC_{50} value. This concentration range also agrees with the micromolar concentrations which have been suggested to be active in the brain (Rowell, 1987). The actual concentration of nicotine which is found in the brain during cigarette smoking is unknown, although the range 0.1 - 0.5 μM nicotine is found in the blood of cigarette smokers (Russell *et al.*, 1980). What proportion of the blood nicotine penetrates the brain is unknown and estimates have been difficult because nicotine is rapidly metabolised. In contrast to these micromolar concentrations, from [^{3}H]nicotine and [^{3}H]ACh binding studies to rat brain membranes, high affinity agonist binding sites have been shown to have nanomolar affinity constants (Clarke *et al.*, 1985a). However, in binding assays, the long incubation periods used (approximately 1 h) may cause the high affinity nicotine binding sites to transform to a desensitised state (Romano and Goldstein, 1980; Schwartz and Kellar, 1983).

At a concentration of 1 μM, nicotine evoked the release of well defined consistent responses (Fig. 4.3 and Table 4.4) and at this agonist concentration nomifensine (50 μM) had no effect on the nicotine response indicating that little if any reuptake of released radiolabelled transmitter occurred. Micromolar concentrations of agonists were therefore used to study the effect of antagonists on agonist-evoked transmitter release.

a) Stereoselectivity of the nicotine response

Many of the classical pharmacological receptors show stereoselectivity, that is stereoisomers of drugs act at the receptors

with different affinities. Using the perfused striatal synaptosome preparation the presynaptic nAChR facilitating DA release displays this receptor characteristic, the heteroreceptor being highly selective for the naturally occurring (-) isomer (Fig. 4.4). The 100 fold difference in the relative potencies of the (+) and (-) isomer was observed when 1 μM (-)-nicotine was used as the control response. To observe an equivalent response using (+)-nicotine, a concentration of 100 μM was required. This 100 fold difference in sensitivity could mean that (+)-nicotine is completely inactive, then a 1% contamination of the (+)-nicotine with (-)-nicotine could account for these results (Barlow *et al.*, 1972). Absolute pure (+)-nicotine is difficult to chemically prepare (Barlow and Hamilton, 1965) and this may explain the conflicting reports in the literature of the relative potencies of the two isomers (see Wonnacott, 1985). However, using (+)- nicotine of high purity (97.8%) Barlow and Hamilton (1965) reported that the stereoselectivity of different tissue preparations varied greatly; the relative potency of (-)-nicotine was greatest at receptors in ganglia.

The non-linear extremities of the nicotine dose resonse curve (Fig. 4.1) could explain the levelling off of the (+)-nicotine curve in Fig. 4.4 and hence the lower stereoselectivity observed at low nicotine concentrations. Similarly, at high saturating concentrations of nicotine (> 100 μM) the same effect was observed. Furthermore, this would explain the failure of Connelly and Littleton (1983) to show stereoselectivity of nicotine-evoked release of [^{3}H]DA from whole brain synaptosomes using nicotine concentrations greater than 1 mM.

The observed stereoselectivity of the heteroreceptors is consistent with the high affinity [^{3}H]nicotine binding site in rat brain (Sloan *et al.*, 1985; Abood *et al.*, 1985; Wonnacott, 1986). In contrast, central cholinergic binding sites labelled with [^{125}I] α-BGT display little stereoselectivity; both (+) and (-) displacing the toxin binding with similar potency (Wonnacott, 1986). On the basis of low stereoselectivity the [^{125}I]α-BGT binding site in rat brain resembles the neuromuscular and *Torpedo* type of nAChRs, whereas the [^{3}H]nicotine binding site shares the stereoselective characteristics of ganglionic type of receptors (Wonnacott, 1986).

4.4.3 Comparison of the release of [^{3}H]DA from striatal synaptosomes evoked by nicotinic agonists

The observed equipotency of 1 μM concentrations of ACh, (-)-nicotine and the ganglionic agonist DMPP is in agreement with previous reports. Rowell and Winkler (1984) and Westfall *et al.*, (1983a) showed that (-)-nicotine and DMPP were equally effective in their transmitter releasing actions. DMPP has also been shown to be of a similar potency to ACh in releasing [^{3}H]ACh from synaptosomes of the myenteric plexus (Briggs and Cooper, 1982). There are however few reports of cytisine-evoked transmitter release. Instead, the central effects of cytisine have been demonstrated using binding and behavioural studies. Romano and Goldstein (1980) reported that cytisine and nicotine were more effective at displacing [^{3}H]nicotine binding to rat brain membranes than were lobeline or DMPP. Similarly, Clarke *et al.* (1984) reported a similar order of potency although cytisine was six times more effective than (-)-nicotine. In contrast, behavioural studies in

mice demonstrated that (-)-nicotine and DMPP were more potent convulsants than lobeline, (+)-nicotine and cytisine (Caulfield and Higgins, 1983). However, the results of many behavioural studies are sometimes misleading because cytisine does not pass readily into the brain (Romano *et al.*, 1981). The relative central potency of various drugs given systemically therefore depends on their respective permeabilities into the brain.

The preliminary experiments carried out with the ganglionic agonists lobeline and coniine (Table 4.1) showed that they were slightly less potent than the other agonists discussed above. This agrees with the low relative potency of lobeline in the ganglia (Taylor, 1985a) but disagrees with the release studies carried out by Sakurai *et al.* (1982). This group demonstrated that the ganglionic agonists lobeline, coniine and sparteine stimulated [^{3}H] DA release from striatal tissue with greater efficacy than nicotine. However, the concentration of agonists used in these studies was high (> 50 μM) and the tissue was incubated with each drug for long periods (12 min). Under these conditions it is possible that nicotine causes more desensitization of the receptors than the other ganglionic agonists (Taylor, 1985a).

At the lower concentrations used in this thesis comparison of the S_2/S_1 and S_3/S_1 values for (-)-nicotine, DMPP and cytisine (Table 4.4) showed that the three agonists were similar. At a concentration of 1 μM the S_2/S_1 value for DMPP was only slightly higher than that of nicotine or cytisine. The S_3/S_1 value for DMPP was also slightly higher than the others, although this was not statistically significant. This may indicate that DMPP is more effective than (-)-nicotine at releasing [^{3}H]DA from striatal

synaptosomes. This would agree with the potency of DMPP in the ganglia being three times that of nicotine (Taylor, 1985a). However, to confirm this suggestion a full range dose-response curve for DMPP is required.

4.4.4 The effect of nicotinic antagonists on evoked [^{3}H]DA release from striatal synaptosomes

The effect of a range of nicotinic antagonists on agonist-evoked [^{3}H]DA release was tested to determine whether i) the agonists were acting specifically at nAChR; ii) if the nicotinic heteroreceptor could be classified as either C_6 or C_{10} and iii) to gain an insight into the functioning of the receptor.

A useful advantage of the final perfusion schedule is that information about the recovery of the nicotinic response after exposure of the synaptosomes to an antagonist can be obtained by measuring the S_3 response. The protocol is also flexible in that different pre-exposure times may be used (e.g. to study the effect of toxins). As mentioned in Section 3.1.1 (p.**110**) in studies where the effects of drugs are observed on either electrical or K^+-evoked transmitter release it is quite common to repeatedly stimulate slice preparations. In contrast, repetitive nicotine-evoked release of [^{3}H]DA from striatal slices has only been reported by Giorgueff *et al.* (1976) and there are no reports of repeatedly stimulated synaptosomal preparations.

The perfusion schedule described in this thesis is similar to that used by Giorguieff *et al.* (1976) which can be summarised as follows: after a 15 - 20 min washout period the slices were stimulated twice (for 7.5 min) with a 20 min interval separating

the stimuli. The effect of an antagonist such as mecamylamine on ACh (10 μM) evoked release was studied by introducing the antagonist into the perfusion medium 15 min before the second stimulation and continuing throughout the last stimulation (Giorguieff *et al.*, 1976). There are some notable differences in the timing, but the overall approach is similar. The results obtained in this thesis have therefore been compared, where applicable, to the findings of Giorguieff *et al.*, (1976).

The period of pre-exposure of the synaptosomes to the antagonist before the second stimulation (S_2) was usually 10 min, which is also the time period reported by Ascher *et al.* (1979) to be adequate to study the effect of nicotinic antagonists on parasympathetic neurones. Longer pre-exposure times were also used (e.g. for NSTX, α-BGT, H_{12}HTX) and these will be discussed separately below. An important control was the K^+-evoked release in the presence of the nicotinic antagonists. This provided evidence for the specificity of the nicotinic response and in all cases the K^+-evoked release in the presence of an antagonist was > 80% control response.

i) C_{10} antagonists

<u>α-BGT</u>

The action of α-BGT on nicotine-evoked release of [^{3}H]DA from striatal synaptosomes was of particular interest because of the mixed reports of its action in the CNS (Section 1.3.2, p. **29**). Antagonism of the normal nicotine (10 μM) response was only observed after prolonged exposure (30 - 55 min) of the synaptosomes

to the toxin (0.125 μM) suggesting that α-BGT may require time to bind before it is effective.

Futerman *et al.* (1982) reported that α-BGT (0.1 μM) caused a 20% inhibition of nicotine (100 μM) evoked release of [^{3}H]DA from striatal synaptosomes (the release measured using static incubation rather than perfusion) which is consistent with the work of de Belleroche and Bradford (1978) who showed a 38.7% inhibition of [^{14}C]DA release by 0.18 μM α-BGT (using a perfused striatal synaptosomal preparation). However, Misgeld *et al.* (1980) found 0.6 μM α-BGT to be without effect on the intrinsic cholinergic excitation in striatal slices and Mills and Wonnacott (1984) found the inhibition of nicotine (100 μM) evoked release of [^{3}H]DA from perfused striatal synaptosomes by α-BGT (0.001 - 0.2 μM) to be inconsistent and ineffective at concentrations greater than 0.1 μM.

The differences in the literature may be attributed to the different experimental procedures employed. Another possibility is that some of the α-BGT preparations are contaminated with κ-BGT (see p. 12) which is active at both C_6 and C_{10} receptors. Because relatively high concentrations of α-BGT were employed in the perfusion experiments (compared with the nanomolar concentrations reported for the affinity constant of [^{125}I]α-BGT binding to rat brain membranes, see Schmidt *et al.*, 1980) a small amount of contamination (e.g. 1%) of α-BGT could account for the observed partial antagonism.

The lack of effect of α-BGT in striatal preparations is consistent with its lack of effect in ganglia (Brown and Fumigelli, 1977; Ascher *et al.*, 1979) and the adrenals (Kilpatrick *et al.*, 1981). α-BGT is also ineffective at displacing [^{3}H]nicotine binding

to rat brain membranes (Rapier *et al.*, 1985) indicating that the nicotinic heteroreceptor may be similar to the high affinity [^{3}H] nicotine binding site as well as C_6 receptors.

D-tubocurarine

The ineffectiveness of d-tubocurarine (10 μM) in blocking 1 μM DMPP-evoked [^{3}H]DA release from striatal synaptosomes is in complete contrast to the almost total antagonism reported by Giorgueff-Chesselet *et al.* (1979) using a lower concentration of antagonist (5 μM and 1 μM nicotine . Antagonism of 10 μM DMPP-evoked [^{3}H]ACh release from ileal synaptosomes by d-tubocurarine at concentrations less than 10 μM has also been reported by Briggs and Cooper (1983). These workers report an increase in the basal release when concentrations greater than 10 μ M are employed. This accords with the reported excitation of central neurons by d-tubocurarine (Krnjević, 1975). It is therefore possible that the concentration of d-tubocurarine used in the experiments reported in this thesis was too high, although introduction of d-tubocurarine alone did not notably change the basal rate of release compared with the control system.

Decamethonium

Decamethonium has been reported to be a more potent inhibitor of both [^{3}H]nicotine binding (Romano and Goldstein, 1980) and [^{125}I]α-BGT binding (Schmidt, 1977) to rat brain membranes than hexamethonium but the same relative potency was found in the perfused striatal synaptosomal preparation. The failure of the nicotine S_3/S_1 responses to recover after exposure to decamethonium

(Table 4.10) is possibly a result of the complex nature of the antagonism caused by decamethonium (see p.14). Decamethonium is therefore probably not a useful antagonist to study the nicotinic regulation of DA release in the striatum.

Dihydro-β-erythroidine

DHβE was one of the most potent antagonists of [^{3}H]DA released from striatal synaptosomes evoked by nicotinic agonists, its action being readily reversible (Table 4.10). These results agree with the potency of DHβE in displacing both [^{3}H]nicotine (Clarke *et al.*, 1984) and [^{125}I]α-BGT binding (Schmidt, 1977) to rat brain membranes, indicating that DHβE may act directly at the agonist binding site. Although there are no other reports of DHβE preventing transmitter release evoked by nicotinic agonists, using perfusion techniques, electrophysiological studies carried out by McLennan and Hicks (1978) and Lichtensteiger *et al.* (1982) have shown that ACh-evoked excitations are blocked by application of DHβE. However, in view of the equipotency of DHβE at both C_6 and C_{10} nAChR (Megirian *et al.*, 1955) the usefulness of DHβE as a specific ligand for differentiating central nAChR is limited.

ii) C_6 antagonists

Neosurugatoxin

NSTX is highly specific for neuronal nAChR (Hayashi *et al.*, 1975) and is extremely potent at nanomolar concentrations (Hayashi *et al.*, 1984). However, biochemical and pharmacological studies have been limited because of the scarcity of the toxin and the difficulties encountered in its purification and storage

(Kosuge *et al.*, 1982). Indeed, the early reports of the potent antagonist 'surugatoxin' (Hayashi and Yamada, 1975; Brown *et al.*, 1976; Ascher *et al.*, 1979) have now been attributed to contamination of the preparation with NSTX (Kosuge *et al.*, 1982).

The action of NSTX in preventing DMPP and nicotine evoked release of [^{3}H]DA from striatal synaptosomes (Fig. 4.6) agrees with its action in displacing high affinity [^{3}H]nicotine binding from rat forebrain membranes (Hayashi *et al.*, 1984; Rapier *et al.*, 1985; Yamada *et al.*, 1985) and also its effect on autonomic ganglia (Ascher *et al* 1979; Hayashi *et al.*, 1975). In all cases NSTX is at least 3 orders of magnitude more potent than hexamethonium. The same relative potency has also recently been shown in behavioural studies (Yamada *et al.*, 1986) in which NSTX prevented nicotine-induced antinociception.

The observed reversibility of the antagonism caused by NSTX in the striatal synaptosomal preparation is also in agreement with its action at auton[illegible] of [illegible] *et al.*, 1979). However, more recent studies by Yamada *et al.* (1986) indicate that the blockade of [^{3}H]nicotine binding sites by NSTX in rat forebrain membranes is irreversible, and the group conclude that NSTX acts as a noncompetitive antagonist. However, the classification of Yamada *et al.* (1986) is different from that used in this thesis (see p.**10**) where a noncompetitive antagonist is defined as acting at a region of the receptor other than the agonist binding site. Using this classification, the results of Yamada *et al.* (1986) would be interpreted as irreversible competitive binding.

Hexamethonium

The observed lack of effect of hexamethonium (10 μM) on DMPP (1 μM) evoked release of [^{3}H]DA from striatal synaptosomes is consistent with the work of Futerman *et al.*, 1982; Sakurai *et al.*, 1982; Connelly and Littleton, 1983; Marien *et al.*, (1983). However, there are reports that hexamethonium causes partial antagonism of transmitter release evoked by nicotinic agonists (Westfall, 1974; 1983a; de Belleroche and Bradford, 1978; Giorguieff *et al.*, 1976; Rowell and Winkler, 1984). These discrepancies are difficult to explain because of the differences in experimental procedures used such as the concentrations of nicotinic agents and the period of stimulation. The results are also difficult to compare because of the reported increase in basal release caused by hexamethonium at concentrations greater than 100 μM (Giorguieff *et al.*, 1976). In behavioural studies hexamethonium is also less effective than other antagonists (e.g. pempidine and mecamylamine) in preventing nicotine-evoked responses (Romano *et al.*, 1981; Caulfield and Higgins, 1983; Stolerman *et al.*, 1983; Yamada *et al.*, 1986), although this is because hexamethonium does not readily penetrate the blood-brain barrier.

In binding studies hexamethonium is a poor inhibitor of [^{3}H] nicotine and [^{125}I]α-BGT binding to rat brain membranes (e.g. Abood *et al*,. 1980; Schmidt, 1977) although hexamethonium is an effective competitive antagonist at ganglionic nAChR (Brown, 1980). Recently, hexamethonium has been shown to exert its action by blocking the receptor ionophore when it is open (Ascher *et al.*, 1979; Rang, 1982; Gurney and Rang, 1984). Antagonism by hexamethonium therefore occurs after the agonist has bound. This

could explain the ineffectiveness of hexamethonium in the revised perfusion system in which a short period (40 s) of stimulation with a low concentration of agonist was used. Using a longer stimulation period and/or a higher nicotine concentration inhibition of the nicotinic response might be observed. This would explain the antagonism by hexamethonium reported by Giorgueff *et al.* (1976).

Mecamylamine

Mecamylamine is similar to hexamethonium in that it is a poor inhibitor of [^{125}I]α-BGT binding (Schmidt, 1977) and also [^{3}H] nicotine and [^{3}H]ACh binding (Abood *et al.*, 1980; Schwartz *et al.*, 1982). However, in contrast to hexamethonium, in the perfused synaptosomal preparation 5 μM mecamylamine was an effective antagonist of [^{3}H]DA release evoked by either nicotine, DMPP and cytisine (Table 4.5). These results agree with the earlier reports from the laboratory (Mills and Wonnacott, 1984) and also the antagonism of nicotine or carbamylcholine induced release of [^{3}H] noradrenaline from hypothalamic synaptosomes (Yoshida *et al.*, 1980). The blockade of the nicotinic response is also in agreement with the work of Giorguieff *et al.* (1976), who showed that mecamylamine at a concentration of 10 μM almost completely blocked ACh (10 μM) evoked release of [^{3}H]DA from striatal slices. This suggests that a higher concentration of mecamylamine should be tested in the synaptosomal system. The lack of recovery of the S_3 response after exposure to mecamylamine (Table 4.10) suggests that the inhibition is only slowly reversible. This agrees with the work of Ascher *et al.* (1979) who reported that a 60 min wash period was required after exposure to mecamylamine to restore activity of the

rat ganglion preparation, in contrast to the 30 min wash period required for the other nicotinic antagonists tested (Ascher *et al.* 1979). At parasympathetic ganglia mecamylamine acts as both a competitive and noncompetitive inhibitor, and this mixed type of antagonism, although much lower, is also displayed at C_{10} nAChR (Brown, 1980). Indeed recent studies by Varanda *et al.* (1985) have shown that mecamylamine acts as a noncompetitive antagonist of C_{10} nAChR by modulating the receptor ionophore (as shown by the inhibition of $[^3H]H_{12}HTX$ binding). In keeping with the nature of the observed antagonism in the perfusion experiments reported in this thesis, Varanda *et al.* (1985) found that exposure of the frog sartorius muscle preparation for 30 min to 10 μM mecamylamine caused complete blockade of the neuromuscular transmission and a 1 h period of washing was required to restore activity.

The inhibitory action of mecamylamine accords with the numerous reports of its action in the CNS. Morgan and Pfeil (1979) and Roth *et al.* (1982) have shown that nicotine induced depletion of rat brain catecholamines is prevented by mecamylamine, which is in keeping with the action of mecamylamine in reducing nicotine-evoked brain activity (London *et al.*, 1985b). Similarly, the excitation of central neurones by iontophoretic application of nicotine has been blocked by mecamylamine (McLennan and Hicks, 1978; Clarke *et al.*, 1985b).

Mecamylamine is also effective at preventing many behavioural responses (e.g. Romano *et al.*, 1981; Stolerman *et al.*, 1983; Caulfield and Higgins, 1983; Yamada *et al.*, 1986).

Pempidine

Pharmacologically, pempidine is similar in action to mecamylamine (Taylor, 1985a). It is a poor inhibitor of [^{3}H] nicotine and [^{125}I]α-BGT binding to rat brain (e.g. Romano and Goldstein, 1980; Wonnacott, unpublished) and proved to be an effective inhibitor of nicotine and DMPP-evoked [^{3}H]DA release from striatal synaptosomes (Table 4.5). These results agree with the action of pempidine in preventing nicotine-evoked [^{3}H]DA release from striatal slices (Giorguieff-Chesselet *et al.*, 1979) and [^{3}H]DA release from whole brain synaptosomes (Connelly and Littleton, 1983). Although pempidine has not been used as much as mecamylamine it has been shown to block nicotine-evoked behavioural responses (Romano *et al.*, 1981; Caulfield and Higgins, 1983; Yamada *et al.*, 1986).

Chlorisondamine

In a preliminary experiment chlorisondamine (5 μM) was found to block nicotine, DMPP and K^{+}-evoked [^{3}H]DA release from striatal synaptosomes by about 20%, suggesting that it is not a specific inhibitor of the nicotinic response. This agrees with the ineffectiveness of chlorisondamine at preventing nicotine-evoked activation of dopaminergic neurones in the substantia nigra pars compacta, a brain region rich in high affinity [^{3}H]nicotine binding sites (Clarke *et al.*, 1984, 1985b).

At parasympathetic ganglia chlorisondamine is a potent noncompetitive antagonist (Brown, 1980) which would account for the inability of chlorisondamine to prevent [^{3}H]nicotine binding to rat brain membranes (Romano and Goldstein, 1980). However, in the CNS

chlorisondamine has been shown to cause long term blockade of nicotine-evoked behavioural responses (Reavil *et al.*, 1986) suggesting that more detailed studies should be carried out employing chlorisondamine in the perfusion experiments.

iii) Classification of the nicotinic heteroreceptor: C_6 or C_{10}

In agreement with previous reports the nicotinic heteroreceptor modulating DA release in the striatum is sensitive to both C_6 and C_{10} antagonists (see Table 1.2). As already mentioned the classification of nicotinic drugs into either C_6 or C_{10} is not well defined (p. 8), with most compounds acting at both receptors although with different relative potencies. It is therefore not surprising that both C_6 and C_{10} antagonists were effective at preventing the nicotinic facilitation of DA release (Table 4.5).

The action of pempidine, mecamylamine and particularly NSTX in preventing both nicotine as well as DMPP evoked [^{3}H] DA release, provides strong evidence for the nicotinic heteroreceptor being related to the C_6 nAChR. However, the classical ganglionic antagonist mecamylamine also acts at C_{10} receptors (Varanda *et al.*, 1985). This dual action is particularly interesting as mecamylamine has recently been shown at different concentrations to block two very different behavioural responses in mice (Collins *et al.*, 1986), leading to the proposal that mecamylamine may act at two distinct central receptors which may correlate with the high affinity [^{3}H]nicotine binding site and the [^{125}]α-BGT binding site (Collins *et al.*, 1986). The classification of nicotine-evoked responses in the CNS as "ganglionic" on the basis of their

sensitivity to mecamylamine (e.g. Brown, 1979) may therefore be inappropriate.

So far NSTX has only been shown to be effective at C_6 receptors (p. **13**), its potency at blocking nicotine and DMPP-evoked DA release is good evidence for the C_6 nature of the nicotinic heteroreceptor. The ability of NSTX to prevent [^{3}H]nicotine binding but not α-BGT binding to rat brain membranes (see p.**36**) also suggests that the nicotinic heteroreceptor is similar to the high affinity [^{3}H]nicotine binding site. However, the antagonism of the nicotinic heteroreceptor by DHβE and the partial block observed by decamethonium and α-BGT suggest that the presynaptic nAChR is related to C_{10} nAChR. DHβE is however equipotent at C_6 receptors and the weak action of α-BGT may be due to κ-BGT.

In conclusion, the nicotinic heteroreceptor modulating DA release has pharmacological properties characteristic of both C_6 and C_{10} receptors.

4.4.5 The physiological role of the nicotinic heteroreceptor

The studies reported in this thesis confirm the earlier reports (see Table 1.2) that the nicotinic heteroreceptor on striatal dopaminergic nerve terminals enhances transmitter release. The observed stereoselectivity of this response (Fig. 4.4) and the sensitivity to NSTX but not α-BGT suggests that the nicotinic heteroreceptor resembles the C_6 nAChR and the high affinity [^{3}H] nicotine binding site. Binding studies using [^{3}H] ACh have shown that high affinity [^{3}H]ACh binding sites may be equivalent to high affinity [^{3}H]nicotine binding sites (Clarke *et al.*, 1985a). It was therefore of interest to compare the action of the endogenous

compound ACh with that of nicotine. In some experiments carbamylcholine was used to avoid the problems associated with using ACh (see p. **54**).

ACh and carbamylcholine are cholinergic agonists which interact with both mAChR and nAChR. ACh acts equally at both receptors whereas carbamylcholine may have a preference for mAChR (Lucchelli *et al.*, 1986). In order to examine the action of ACh, anticholinesterases were not included in the perfusion medium because of their reported interaction with the nAChR-ionophore (Shaw *et al.*, 1985). ACh was effective at releasing [^{3}H]DA from the perfused synaptosomes and was equipotent with (-)-nicotine., Interestingly, carbamylcholine was of lower efficacy than ACh and nicotine (Table 4.1). This order of relative potency is in keeping with Ascher *et al.*, (1979) who found that nicotine and ACh (in the presence of acetylcholinesterase inhibitors) were more potent than carbamylcholine in the rat ganglion. However, the results are contrary to those of Yoshida *et al.* (1980) who demonstrated that 1 - 10 μM nicotine and carbamylcholine were equipotent at releasing noradrenaline from hypothalamic synaptosomes.

If no hydrolysis of ACh had occurred it would be expected that the response by ACh would be greater than (acting at both facilitatory muscarinic and nicotinic receptors) or equal to (acting mainly on nAChR) the response to nicotine. To determine whether ACh was interacting with muscarinic or nicotinic or both types of cholinergic receptors, the effect of the nicotinic antagonist DHβE (0.5 μM) and the muscarinic antagonist atropine (0.5 μM) were tested on ACh evoked release of [^{3}H]DA. The radioactivity released by ACh (100 μM) was not inhibited by atropine but was partially

prevented by DHβE (Table 4.11). Similarly, the response by carbamylcholine (100 μM) was sensitive to DHβE but not atropine. No obvious interaction of ACh or carbamylcholine with muscarinic heteroreceptors was therefore observed.

However, the muscarinic agonist oxotremorine (10 μM) was found to release [^{3}H]DA from the perfused striatal synaptosomes. The action of oxotremorine on basal release of DA from striatal tissue although previously observed (Giorguieff *et al.*, 1977; Bartholini, 1980; Futerman *et al.*, 1982) has not been well characterised. Its facilitatory action is usually measured on stimulated release of radiolabelled transmitter from perfused slices (Lehmann and Langer, 1982; de Belleroche and Gardiner, 1982) and is associated with muscarinic heteroreceptors. Recently, oxotremorine has been shown to be a partial nicotinic agonist in frog rectus abdominus and cultured chick myotubes. (Lucchelli *et al.*, 1986; Haggbald *et al.*, 1985) but it is of very low potency (Lucchelli *et al.*, 1986). The possibility of the observed release of oxotremorine being a result of the interaction with nAChRs is therefore doubtful. More likely is the interaction of oxotremorine with muscarinic heteroreceptors as reported by Giorguieff *et al.*, (1977), who showed that at 10 μM it was 60 times less effective at eliciting transmitter release than ACh of an equal concentration. The release by oxotremorine was also completely blocked by the muscarinic antagonist atropine (1 μM) (Giorguieff *et al.*, 1977) confirming the muscarinic nature of the response. Further studies using a range of cholinergic antagonists would help characterise the observed effects of oxotremorine described in this thesis.

In view of the anticipated hydrolysis of ACh, the action of choline was tested. Choline (10 μM) was found to evoke the release of [^{3}H]DA from striatal synaptosomes although the response was about half that observed with ACh (10 μM) (Table 4.1). These results are contrary to those of Giorguieff *et al.* (1976) who found that choline (10 μM) was without effect on [^{3}H] DA release from striatal slices. This difference might reflect differences in the sensitivities of the perfusion systems employed and/or the tissue preparations used.

In the periphery choline interacts with both mAChR and nAChR (Dale, 1914), although choline is much less potent than ACh. Evidence for choline acting at a functional nAChR in the PNS was provided by Sorimachi and Nishimura (1982) who showed that 100 mM choline induced the secretion of catecholamines from cat adrenal medulla which was comparable to that elicited by an ACh concentration of 0.02 mM, showing that there is a large difference in the relative potencies of choline and ACh. In contrast, in the CNS, choline has been shown to be an effective agonist or "false transmitter" (Krnjević and Reinhardt, 1979; Ropert and Krnjević, 1982), its action being about 5 times weaker than ACh. The observed choline-evoked release of [^{3}H]DA, whether it be via muscarinic or nicotinic heteroreceptors, is therefore of particular interest, especially if it is also applicable at cholinergic synapses (via autoreceptors) where high concentrations of choline would be expected. This may be of relevance to the treatment of Alzheimer's disease in which choline is often administered in an attempt to enhance the levels of ACh in the hippocampus (Growdon and Wurtman, 1983).

In the brain nicotine is not an endogenous compound. There must therefore be a naturally occurring substance which interacts specifically with central nAChR. It is possible that the ligand is ACh, although the possibility remains that it is one of the 'endogenous' ligands that prevents the binding of[^{3}H]nicotine or [^{125}I]α-BGT to rat brain membranes (Shershen *et al.*, 1984; Perry *et al.*, 1986; Quik, 1982; see p.**105**). The method of perfusion would however ensure that any such ligand is washed away. It is possible that *in vivo* the presynaptic receptors are activated by 'cotransmitters' released together with the main transmitter (Bartfai, 1985). Both substance P agonists and antagonists have been shown to inhibit ion conductance through nAChR on PC12 cells (Eardley and McGee, 1985). Morphine and (Met^{5})enkephalin have also been shown to inhibit the release of [^{3}H]DA or endogenous DA following activation of nicotinic cholinergic receptors, whereas K^{+} or field stimulation is unaffected (Westfall, 1983b).

The latter observation is particularly interesting because opioid peptides have been shown to reduce nicotine-evoked release of catecholamines from the adrenal medulla (e.g. Dean *et al.*, 1982). These interactions may be purely artefactual or of physiological relevance.

4.4.6 Mechanism of nicotinic modulation of transmitter release

From electrophysiological studies the mechanism of action of the nAChR at the neuromuscular junction has been well characterised (p. **3**) and it has been shown that activation of these nAChR leads to an influx of Na^{+} through an associated ion channel. At the nerve terminal presynaptic nAChR may function by a similar mechanism,

with an influx of Na^+ ions resulting in local depolarisation and the opening of Ca^{2+} channels. Indeed, using a striatal synaptosomal system the release of [^{3}H]DA has been shown to be associated with the influx of Ca^{2+} (Drapeau and Blaustein, 1983).

It is generally accepted that extracellular Ca^{2+} is essential for transmitter release, although Ca^{2+}-independent release has been reported. The observed partial Ca^{2+}-dependency on nicotine-evoked [^{3}H]DA release from synaptosomes (Table 4.3) agrees with the work of Connelly and Littleton (1983), and Rowell and Winkler (1984). Complete Ca^{2+}-dependency was not expected because of the presence of endogenous amounts of Ca^{2+} (see the Ca^{2+} dependency of basal release, p. **173**). However, total Ca^{2+} dependency has been reported for nicotine stimulated release of [^{3}H] DA from striatal slices (Westfall *et al.*, 1974a; Giorguieff *et al.*, 1977; 1979) whereas other groups (Arqueros *et al.*, 1978; Marien *et al.*, 1983) have demonstrated complete Ca^{2+}-independency. This lack of requirement for external Ca^{2+} was observed when high nicotine concentration (3 - 5 mM) were used suggests that at such concentrations the release is largely non-specific (Arqueros *et al.*, 1978). This agrees with the greater amount of Ca^{2+}-dependent release observed using low concentrations of stimulus (see Table 4.3) in the synaptosomal perfusion system and reinforces the importance of using low concentrations of agonists in release studies. The Ca^{2+}-dependency of K^+ (28 mM) and veratridine (10 µM) evoked [^{3}H]DA release from striatal synaptosomes was about the same (although veratridine was slightly higher) than that of low (1 µM) concentrations of either DMPP or nicotine stimulated release. This suggests that the nicotinic agonists are acting via the normal

physiological process by which neurotransmitters are released in response to depolarisation.

The inhibition of the nicotine-evoked release of [^{3}H]DA by H_{12}HTX (Fig. 4.7, Table 4.9) suggests that the nicotinic heteroreceptor may have an associated ionophore and agrees with the action of HTX at nAChR on *Electrophorus* electroplaques (Kato and Changeux, 1976; Bartels-Bernal *et al.*, 1983), neuromuscular preparations (e.g. Spivak *et al.*, 1982), cultured muscle cells (Burgermeister *et al.*, 1977) and adrenal medullary cells (Kilpatrick *et al.*, 1980; McKay *et al.*, 1985).

In the CNS,a nAChR-associated ion channel is implicated in the characteristic fast depolarisations recorded in response to the iontophoresis of nicotinic drugs (Krnjević, 1975), and HTX has been shown to block the excitation of cholinoreceptor cortical neurones by ACh (Glavinovic *et al.*, 1974). [^{3}H]H_{12}HTX has been shown to bind to electroplaque membranes (Eldefrawi *et al.*, 1980; Heidmann *et al.*, 1983; McKay *et al.*, 1985) and to chick optic lobe (Betz, 1982). However, the binding data are difficult to interpret because HTX also acts (at higher concentrations) with Na^+ and K^+ channels (Bartels-Bernal *et al.*, 1983). The lack of effect on H_{12}HTX on K^+-evoked [^{3}H]DA release from striatal synaptosomes supports the specificity of H_{12}HTX (at the concentrations used) for the nicotinic ionophore. The EC_{50} value obtained of 5 μM (Fig. 4.7) is the same as the concentration required to produce a 50% blockade in frog sartorius muscle (Spivak *et al.*, 1982) but is higher than the value of 0.8 μM reported by Kato and Changeux (1976) in *Electrophorus electroplaques*. The similarity between the EC_{50} value reported in this thesis and those obtained for peripheral nAChR

suggests that the presynaptic nAChR on dopaminergic neurones acts via a similar ionophore. The time courses observed are consistent with a slow reversible interaction with the nAChR as observed with the electroplaque nAChR. Thus, the preincubation period used of 15 min, after Kato and Changeux (1976), provides evidence for a similarity between the ionophores of the brain and *Electophorus* nAChR, although Bartels-Bernal *et al.* (1983) report a 30 min period is required for maximum blockade. In agreement with Kato and Changeux (1976), a 15 min wash period after partial blockade by H_{12}HTX (Table 4.9) resulted in almost a complete recovery of the nicotinic response.

The inhibition of nicotine-evoked [^{3}H]DA release from striatal synaptosomes by 1 μM ketamine agrees with the action of ketamine at the ion channel of C_{10} nAChR (Maleque *et al.*, 1981) and provides additional evidence for the nicotinic heteroreceptor having an associated ion channel. The antagonism by ketamine was readily reversible (Table 4.9) which agrees with the action of ketamine (5 μM) in preventing nicotine-stimulated release of catecholamines from perfused adrenal glands (Sumikawa *et al.*, 1983). Higher concentrations of ketamine (10 μM) were ineffective at reducing the nicotinic response, however, there are reports that high concentration of agents such as ketamine may have marked effects on membrane fluidity that are distinct from its more specific channel properties (Bradford and Marinetti, 1982).

These results suggest that nicotinic heteroreceptors have an HTX-sensitive ion channel and in this respect closely resemble C_{10} nAChR. Although electrophysiological studies have shown that the C_6 receptor displays channel properties similar to C_{10} receptors

(Ascher *et al.*, 1979; MacDermott *et al.*, 1980) there is no direct evidence for an ionophore being an integral part of the C_6 receptor. Purification of C_6 nAChR has not yet been possible because ganglia are a low source of receptor. However, predictions are being made about its structure using cloning techniques employing PC-12 cells (p.**34**). The presence of the amphipathic region in the amino acid sequence of the isolated PC-12 nAChR subunit suggests that the ion channel may be highly conserved in different classes of nAChR.

CHAPTER 5

CHARACTERISATION OF THE NICOTINIC REGULATION OF [^{3}H]ACh RELEASE FROM HIPPOCAMPAL SYNAPTOSOMES

5.1 INTRODUCTION

The results reported in this thesis so far, have clearly demonstrated the presence of presynaptic nicotinic heteroreceptors in rat brain. However, there is little evidence for nicotinic autoreceptors (p. **41**).

The hippocampus is an attractive region in which to study cholinergic autoregulation because its extrinsic cholinergic innervation is entirely derived from the septum. This cholinergic pathway is known as the septo-hippocampal pathway (see Nicoll, 1985) and is analogous to the nigro-striatal dopaminergic pathway which makes the striatum an ideal brain region in which to study the release of DA.

The hippocampus has been shown to be associated with memory and learning functions (Meck *et al.*, 1984) and the involvement of cholinergic pathways has been implicated (e.g. Davis *et al.*, 1978). The reports that nicotine improves memory and behaviour (Nordberg and Bergh, 1985) therefore makes the hippocampus an obvious area in which to study the effect of nicotine on transmitter release.

In our laboratory Moss and Wonnacott (1985), using the original perfusion system described by Mills and Wonnacott (1984), demonstrated the presence of presynaptic nicotinic autoreceptors in rat hippocampus. Hippocampal synaptosomes preloaded with [^{3}H]choline, were shown to specifically release [^{3}H]ACh in response to either nicotine or DMPP (0.1 - 1 mM). Of particular interest was the mixed pharmacology shown by the autoreceptor; the nicotinic facilitation of [^{3}H]ACh being sensitive to the ganglionic antagonists mecamylamine and pempidine and also the neuromuscular blocking agent α-BGT (Moss and Wonnacott, 1985).

5.2 METHODS

5.2.1 Preparation and perfusion of hippocampal synaptosomes

The brain was removed from one male Wistar rat (200 g) and the hippocampus dissected as described in Fig. 2.1 (p.**64**). Synaptosomes were prepared from the tissue as described for striatal synaptosomes (Section 2.2.1, p. **63**). To radiolabel the transmitter pools, the synaptosomes resuspended in perfusion medium to a protein concentration of 2 - 4 mg/ml, were preincubated for 10 min at 37°C followed by the addition of [^{3}H]choline (31 Ci/mmol, final concentration 0.8 µM choline). Incubation at 37°C was continued for a further 20 min and the uptake determined by washing samples (20 µl) of the suspension by vacuum filtration (Section 2.2.6, p. **71**). The remaining synaptosomes were washed by centrifugation (p.**117**) and samples (200 µl) layered onto the filters in the perfusion system. Perfusion was carried out using the final conditions (Section 3.2.4, p.**137**) except that the perfusion medium contained no pargyline or ascorbic acid. Acetylcholinesterase inhibitors were not included in the medium because of the recent reports of their possible interaction with the nAChR-ionophore (Shaw *et al.*, 1985).

5.2.2 The effect of nicotine and nicotinic antagonists on the release of [^{3}H]ACh from hippocampal synaptosomes

The effect of DHβE (0.5 µM) and α-BGT (0.125 µM) on nicotine (10 µM) stimulated release of radiolabelled transmitter was carried out as described for striatal synaptosomes (Fig. 4.5 (p.**203**) and Table 4.7 (p.**209**) respectively).

The effect of H_{12}HTX (4 μM) was determined by stimulating the hippocampal synaptosomes 3 times with (-)-nicotine (10 μM) at 40 min intervals; H_{12}HTX was introduced into the perfusion medium 15 min before S_2 and continued until 15 min before S_3 at which time washing was continued with normal perfusion medium.

Statistical analysis of all data was performed using a two tailed Students' t-test. A value $P < 0.1$ was considered to be statistically significant.

5.3 RESULTS

5.3.1 Uptake of [^{3}H]choline by hippocampal synaptosomes and nicotine-stimulated release of radiolabelled transmitter

Using the loading conditions described in the Methods section above, the hippocampal synaptosomes accumulated 8.19 ± 0.25% (mean ± SEM, n = 7) of the total [^{3}H]choline present in the incubation medium.

Perfusion of preloaded hippocampal synaptosomes resulted in a perfusion profile (Fig. 5.1) comparable with those obtained using striatal synaptosomes. After a 40 min wash-out period the basal efflux of tritium was almost constant and (-)-nicotine (10 μM) clearly stimulated the release of radioactivity above the baseline. The release was dose-dependent (Fig. 5.2), the response to 100 μM (-)-nicotine being significantly greater ($P < 0.002$) than that obtained with 10 μM (-)-nicotine.

The release of radiolabelled transmitter from hippocampal synaptosomes that were repeatedly stimulated with (-)-nicotine was different from the levels of release obtained using striatal synaptosomes (Table 5.1). At high (100 μM) (-)-nicotine concentrations the release by the 3 stimulations, S_1, S_2 and S_3, remained constant.

5.3.2 The effect of nicotinic antagonists on nicotine evoked transmitter release

a) Dihydro-β-erythroidine

DHβE (0.5 μM) inhibited the (-)-nicotine (10 μM) evoked release of [^{3}H]ACh from the hippocampal synaptosomes by almost 60% and at a higher nicotine concentration (100 μM) approximately 30% of the normal response was prevented (Table 5.1).

Fig. 5.1. Nicotine stimulated release of [^{3}H]ACh from hippocampal synaptosomes.

Hippocampal synaptosomes (200 µl, 0.4 mg protein/filter) preloaded with [^{3}H]choline (0.8 µM) were perfused using the final perfusion conditions (Section 3.2.4, p.137). After a 40 min wash-out period the synaptosomes were repeatedly stimulated with 100 µl pulses of (-)-nicotine (10 µM) at 40 min intervals. Arrows indicate time of stimulation.

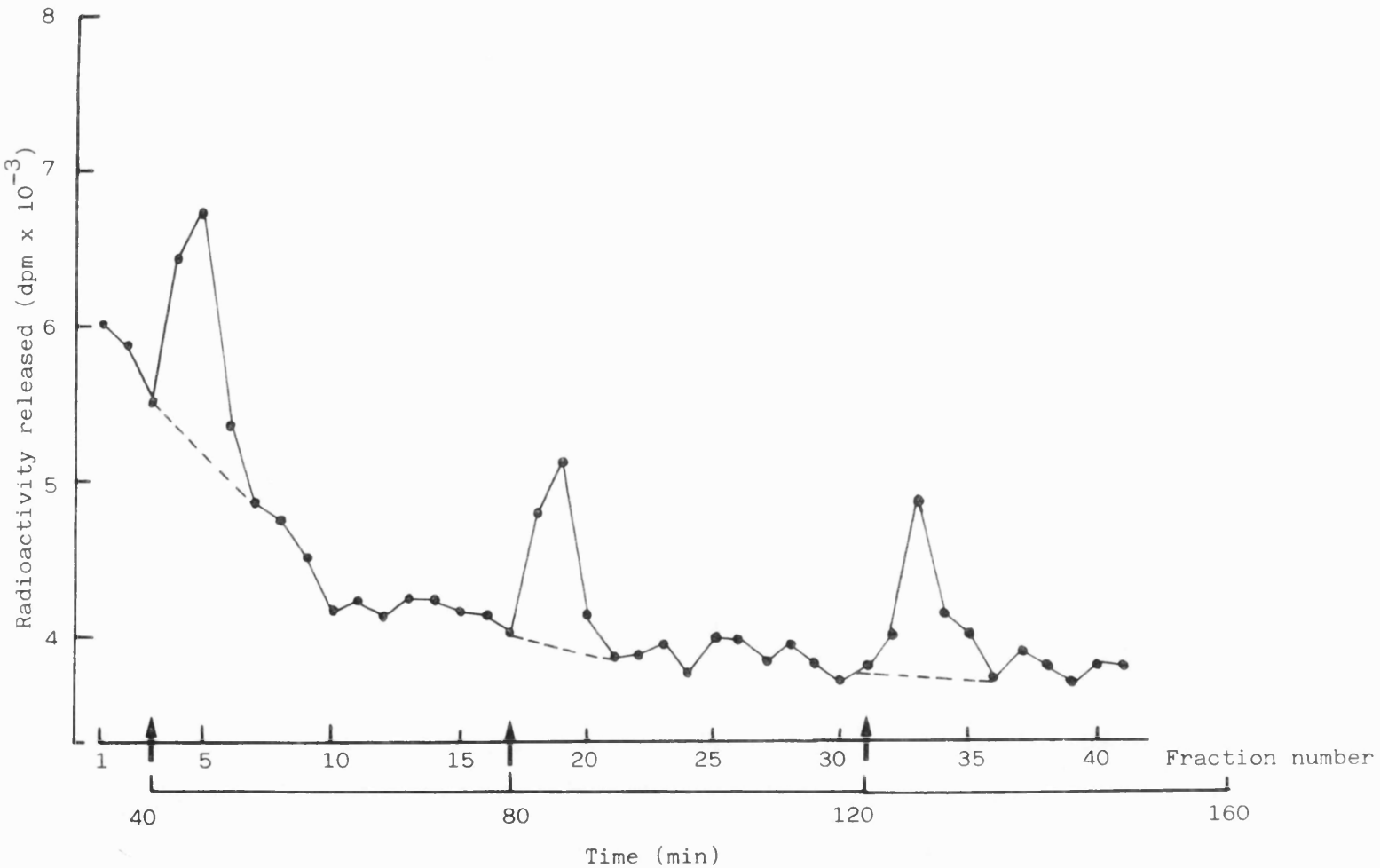


Fig. 5.1. Nicotine stimulated release of [^{3}H]ACh from hippocampal synaptosomes.

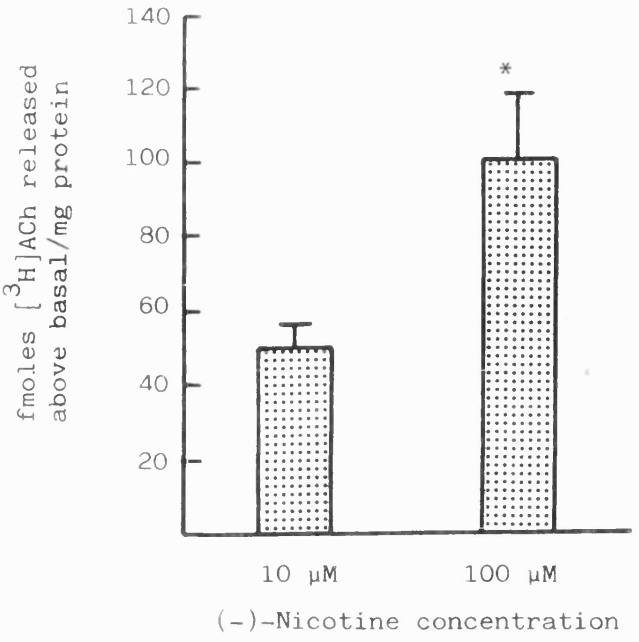


Fig. 5.2. Nicotine-evoked release of [^{3}H] ACh (above spontaneous release) from hippocampal synaptosomes. Results expressed as mean ± SEM (n > 4).

* indicates that the value is significantly greater than the release by 10 µM nicotine, with $P < 0.002$.

Table 5.1. Comparison of repetitive nicotine-stimulated release of [^{3}H]DA from striatal synaptosomes and of [^{3}H]ACh from hippocampal synaptosomes.

Synaptosomal preparation	(-)-Nicotine concentration (μ M)	DHβE(0.5 μM) present during S_2	S_2/S_1 (%)	S_3/S_1 (%)
Striatal	10	-	84 ± 6.8	59.1 ± 5.5
Hippocampal	10	-	88 ± 3.4	76.5 ± 2.1*
Hippocampal	10	✓	37 ± 4(n=2)	ND
Striatal	100	-	38 ± 2.9	19.5 ± 2.9
Hippocampal	100	-	82.5±4.3**	105.8±6.8**
Hippocampal	100	✓	61.5±0.5(n=2)	ND

Synaptosomes preloaded with radiolabelled transmitter were repeatedly stimulated with (-)-nicotine at 30 min intervals. The response by the second (S_2) and third (S_3) pulses were expressed as a percentage of the initial response (S_1). The effect of DHβE (0.5 μM) was tested on nicotine stimulated release of [^{3}H]ACh from hippocampal synaptosomes as described in Fig. 4.5. The S_2 response in the presence of DHβE was calculated as a percentage of S_1. Results are expressed as mean ± SEM (n > 4) and mean ± range (n=2).

* indicates that the value is significantly greater than the S_3/S_1 striatal value with $P < 0.05$.

** indicates that the value is significantly greater than the corresponding striatal value with $P < 0.0005$.

b) α-Bungarotoxin

Introduction of the medium containing α-BGT resulted in a peak equivalent to about 20% of S_1 (nicotine, 10 μM) which spread over 2-3 fractions. The action of α-BGT (0.125 μM) on (-)-nicotine (10 μM) stimulated release was similar to that observed using striatal synaptosomes (p.**209**). After 55 mins exposure to α-BGT a reduction in the normal response of approximately 30% was observed (Table 5.2).

Table 5.2. The effect of α-BGT (0.125 μM) on nicotine (10 μM) stimulated release of [^{3}H]ACh from hippocampal synaptosomes

Perfusion details	S_2/S_1 (%)	S_3/S_1 (%)
normal (control)	94.5 ± 7.5	76.5 ± 3.2
+ α-BGT (0.125 μM)	84 ± 10	55 ± 1.5

Hippocampal synaptosomes preloaded with [^{3}H]choline were perfused using the final perfusion conditions and stimulated 3 times with (-)-nicotine (10 μM) at t = 40, 95, 120 min. In the test system at t = 65 min, α-BGT (0.125 μM) was introduced into the perfusion medium and continued until the end of the perfusion. The release of radioactivity (above the spontaneous level) by the second and third stimulations (S_2 and S_3) were expressed as a percentage of the initial response (S_1).

Results expressed as mean ± range (n = 2).

c) Perhydrohistrionicotoxin

Stimulation of hippocampal synaptosomes with 10 μM (-)-nicotine at 40 min intervals (instead of 30 min) gave S_2/S_1 and S_3/S_1 values of 89.1 ± 4.4 and 77.4 ± 2.5 respectively, (results expressed as mean ± SEM, n = 4). These values lie within the ranges obtained for stimulation at 30 min intervals (Table 5.1).

$H_{12}HTX$ (4 μM) blocked the normal 10 μM nicotine S_2/S_1 response by about 25% (Table 5.3). This antagonism was shown to be reversible after 15 min wash with normal perfusion medium.

Table 5.3. The effect of $H_{12}HTX$ (4 μM) on (-)-nicotine (10 μM) stimulated release of radiolabelled transmitter from hippocampal synaptosomes.

Stimulus	S_2/S_1 (%)	S_3/S_1 (%)
(-)-nicotine 10 μM	75.5 (4)	89.8 (2)

Perfused synaptosomes were stimulated 3 times at 40 min intervals and $H_{12}HTX$ (4 μM) introduced into the perfusion medium 15 min before S_2 and continued until 15 min before S_3.
The S_2/S_1 and S_3/S_1 values were calculated for the test systems and the result expressed as the percentage of analogous S_2/S_1 values obtained in control systems.
Results expressed as the mean of (n) determinations with a variance of <10%.

5.4 DISCUSSION

5.4.1 Perfusion of hippocampal synaptosomes

These preliminary results demonstrate the existence of nicotinic autoreceptors on hippocampal cholinergic nerve terminals and confirm the earlier report from the laboratory by Moss and Wonnacott (1985). The facilitatory action of the nicotinic autoreceptor in the hippocampus is consistent with the nicotinic regulation of [^{3}H]ACh release in the cortex (Rowell and Winkler, 1984) and the myenteric plexus (Briggs and Cooper, 1983). The conditions for labelling the synaptosomes was based on previous studies in the laboratory (Moss and Wonnacott, personal communication) and the level of choline uptake agrees with other studies (see Jope, 1979). The lower level of uptake than found with [^{3}H]DA (using striatal synaptosomes, p.**87**) and the incomplete acetylation of choline to form ACh (approximately 70%, see Wonnacott and Marchbanks, 1976) resulted in lower levels of tritium released from the perfused synaptosomes (Fig. 5.1). This necessitates the higher concentration of nicotine required to evoke quantifiable tritium release, although concentrations lower than 10 μM were not tested. The nicotine evoked release of [^{3}H]ACh was dose-dependent (Fig. 5.2) which is in agreement with the nicotinic autoreceptors reported by Rowell and Winkler (1984), and Moss and Wonnacott (1985).

In the experiments reported in this chapter no cholinesterase inhibitors were present. This meant that the tritium present in the perfusate probably contained a high proportion of [^{3}H]choline. Experiments carried out by Moss and Wonnacott (198) showed that in the presence of a cholinesterase inhibitor about 75% of the tritium

release in response to 0.1 mM nicotine was [^{3}H]ACh. The nicotine evoked release of tritium (above spontaneous) in the experiments described in this chapter was therefore taken as representing mainly [^{3}H]ACh. Using this assumption the improved sensitivity of the revised perfusion system was shown. The release of [^{3}H]ACh by nicotine (0.1 mM) was about 100 fmoles/mg protein (Fig. 5.2) compared with the release using the original system of 0.2 fmoles/mg protein (Moss and Wonnacott, 1985).

Although the results show that auto- and heteroreceptors are similar in their facilitatory actions of neurotransmitter release, there is a notable difference in the sensitivities of the two receptors (Table 5.1). The S_2/S_1 and S_3/S_1 values for 10 μM and 100 μM nicotine-evoked transmitter release are significantly higher for hippocampal than striatal. However, the striatal and hippocampal preparations are not directly comparable because of the differences in the labelling of the respective transmitter pools. Nevertheless, preloading of the synaptosomes with either the precursor [^{3}H]choline (hippocampal) or the transmitter [^{3}H]DA (striatal) gave similar results in terms of the pharmacology displayed by the auto- and heteroreceptor. This suggests that the precursor and transmitter may label similar transmitter pools within the nerve terminal as already discussed for the use of [^{3}H] tyrosine and [^{3}H]DA in striatal release studies (p.180).

Of interest was the observation that DHβE (the potent antagonist of nicotine-evoked release of [^{3}H]DA from striatal synaptosomes) was an effective inhibitor of 10 μM nicotine-evoked release of [^{3}H]ACh from hippocampal synaptosomes (Table 5.1). The lower antagonism by DHβE found using 100 μM nicotine is consistent

with DHβE acting as a competitive antagonist. Unlike the results of Moss and Wonnacott (1985), complete antagonism by α-BGT was not observed. Instead, partial antagonism of 10 μM nicotine-evoked [^{3}H] ACh release was shown to be identical to that found using striatal synaptosomes (see Tables 4.7 and 5.2). The difference between the results obtained by Moss and Wonnacott (1985) and those reported in this thesis probably reflect differences in the experimental conditions employed, including the high concentrations of nicotine and the longer periods of stimulation used in the earlier studies. The results are therefore not directly comparable. It is possible that the observed transient increase in basal release of 20% S_1 (compared with <5% in the striatal preparation) when α-BGT was introduced into the perfusion medium was also present in the earlier studies but the original system was not sensitive enough to detect such small changes. Whether α-BGT is an effective antagonist of nicotinic function in the hippocampus has therefore still to be confirmed. As mentioned in the striatal work, α-BGT may require time to bind before inhibition is observed (for both striatal and hippocampal synaptosomes 55 min was required to observe partial antagonism). Alternatively, the partial antagonism observed by the relatively high concentrations of α-BGT used, may possibly be attributed to contamination of α-BGT with κ-BGT (see p. 12). α-BGT may therefore not be a suitable antagonist in perfusion studies. Instead, DHβE, pempidine and mecamylamine might be more useful in the further characterisation of the nicotinic autoreceptor. The sensitivity of the nicotinic response to H_{12}HTX (Table 5.3) provides evidence that the nicotine autoreceptor may also contain an ionophore as already suggested by similar

experiments using striatal synaptosomes (p.213). At a concentration of 4 μM, H_{12}HTX inhibited the S_2/S_1 response of 10 μM nicotine in the hippocampal preparation by 25% and after 15 min washing there was a partial recovery. These results are directly comparable with those obtained using striatal synaptosomes (Table 4.9).

These preliminary experiments strongly suggest that the nicotinic facilitatory autoreceptors on hippocampal synaptosomes are sensitive to DHβE, H_{12}HTX and α-BGT. In this respect they resemble the striatal nicotinic heteroreceptors modulating DA release described in Chapter 4. Further studies using a range of nicotinic agonists and antagonists will reveal how similar the autoreceptor is to the heteroreceptor.

OVERVIEW

The synaptosomal procedure described in this thesis is a novel system for studying the release of transmitter from nerve endings. It has been used to study the nicotinic heteroreceptor modulating DA release in the striatum and preliminary experiments employing hipp ocampal synaptosomes have shown that it can be readily adapted to look at other release systems.

The nicotinic heteroreceptor shows properties characteristic of C_6 nAChR and also high affinity [^{3}H] nicotine binding sites. However, C_{10} antagonists are also partially effective in preventing the action of the nicotinic heteroreceptor, indicating a relationship between the central nAChR and both C_6 and C_{10} nAChR. The preliminary experiments reported in Chapter 5 indicate that the nicotinic autoreceptor may be similar to the heteroreceptor. However, more detailed studies are required before analogies can be made.

The sensitivity of the central presynaptic nAChR to H_{12}HTX suggests that these nAChR may have an associated ion channel comparable with their peripheral counterparts and may therefore be structurally related. This agrees with the reported immunological cross-reactivity between antibodies raised against peripheral (C_{10}) receptors and purified central receptors (see p.**32**).

The facilitatory nature of the nicotinic heteroreceptor modulating DA release in the striatum agrees with the reported action of nicotinic heteroreceptors modulating the release of noradrenaline from hypothalamic synaptosomes (Yoshida et al., 1980) and 5-hydroxytryptamine release from striatal slices (Westfall, 1983a). The facilitatory nature of the muscarinic heteroreceptor on

striatal dopaminergic nerve terminals (see Table 1.2) also agrees with the action of the muscarinic heteroreceptor modulating DA release in the nucleus accumbens (de Belleroche and Gardiner, 1985). Both central muscarinic and nicotinic heteroreceptors are therefore similar in their enhancing effect on transmitter release. This is in contrast to the cholinergic modulation of noradrenaline release in the PNS, where the muscarinic response is inhibitory and the nicotinic response excitatory (Muscholl, 1979).

The preliminary experiments reported in this thesis employing hippocampal synaptosomes confirms previous reports that nicotinic autoreceptors are facilitatory (see p.**41**). However, muscarinic autoreceptors have been shown to exert an inhibitory action on transmitter release (see p.**41**).In this way muscarinic and nicotinic autoreceptors provide a dual regulation of ACh release which is analogous to the modulation by α_2 and β adrenergic autoreceptors of noradrenaline release (Langer, 1981).

Future directions

To further characterise central nAChR using perfusion techniques, the perfusion system could be modified. Finer control of the temperature and pressure may reduce the slight day to day variation in release rates. The construction of a glass enclosure similar to that recently described by Minnema and Michaelson (1985) would allow such control.

A more detailed study of the pharmacology of the nicotinic response would help in the classification of the nicotinic heteroreceptor as either C_6 or C_{10}. Changes in the perfusion schedule, such as the length of the pre-exposure and wash-off

periods would give more information about the reversibility of the inhibition caused by an antagonist. The determination of the effect of antagonist concentration on a range of agonist concentrations would give information about the nature of the blockade. However, the classification of central nAChR as either C_6 or C_{10} receptors is complicated by the fact that most C_6 and C_{10} antagonists act at both receptors. The classification of nicotinic antagonists as either C_6 or C_{10} is therefore difficult and demonstrates the limitations of this classification. There is therefore a requirement for ligands specific only for one type of receptor. NSTX may be such a ligand, although detailed studies have not been possible because of its scarcity (p.13). The antagonists mecamylamine, pempidine, chlorisondamine and DHβE may also be useful in the further characterisation of central presynaptic nAChR.

Although the facilitatory action of the nicotinic hetero-receptor was only studied on the basal efflux of [^{3}H]DA, there are also reports that these receptors are functional under depolarising conditions (Table 1.2). Similarly, nicotinic autoreceptors have been shown to be operational under either basal or depolarising conditions (Rowell and Winkler, 1984; Moss and Wonnacott, 1985; Beani et al., 1985). Central presynaptic nAChR may therefore be classified as Type I REC (see p.41). To complement the studies reported in this thesis it would be interesting to look at the effect of nicotinic agonists and antagonists on K^+ (16 mM) evoked transmitter release, employing a slightly modified perfusion protocol.

To further study the mechanism of action of the presynaptic

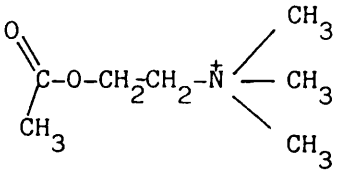
nAChR it would be interesting to determine the Ca^{2+}-dependency of agonist-evoked transmitter release over a range of agonist concentrations. The measurement of $^{45}Ca^{2+}$ entry in response to nicotine stimulation following the methods of Leslie et al. (1985) and Drapeau and Blaustein (1983) may help in this characterisation.

Perfusion experiments using a range of noncompetitive antagonists that are known to interact with the C_{10} nAChR ionophore, such as phencyclidine (Aguayo et al., 1982) or bupivacaine (Aracava et al., 1984) may provide more evidence in support of an ionophore being part of the central presynaptic nAChR. More direct examination of this aspect could be achieved by carrying out ion-flux studies employing a synaptosomal preparation. These studies could be based on the work of Robinson and McGee (1985) and Lindstrom et al. (1980) who have studied ion flux through nAChR on PC-12 cells and reconstituted vesicles respectively.

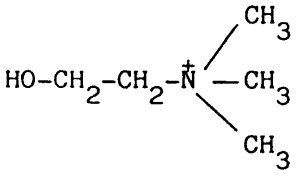
To summarise, this study has shown that functional central nAChR exist which are similar to both C_6 and C_{10} nAChR and a synaptosomal perfusion system has been developed which will help in the further characterisation of central nAChR.

Appendix: Chemical Structures

Nicotinic Agonists



Acetylcholine



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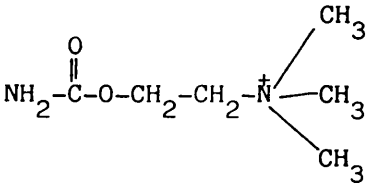

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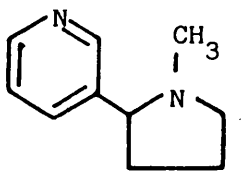

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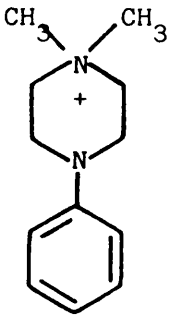
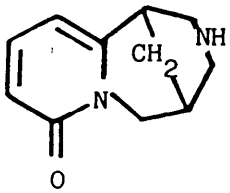


1,1-Dimethyl-4-phenyl piperazinium (DMPP)



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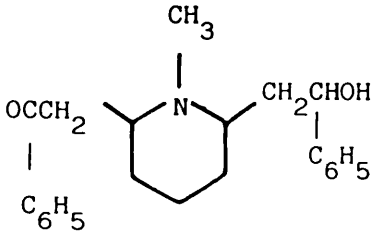
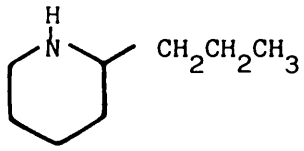
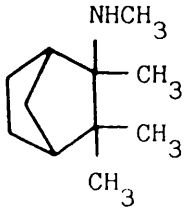


Lobeline

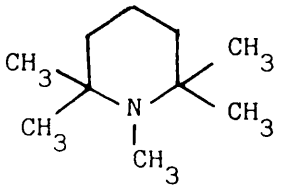


Coniine

Nicotinic Antagonists



Mecamylamine



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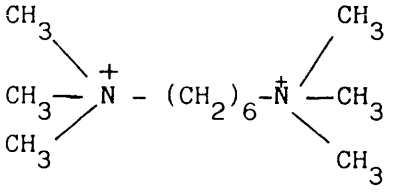

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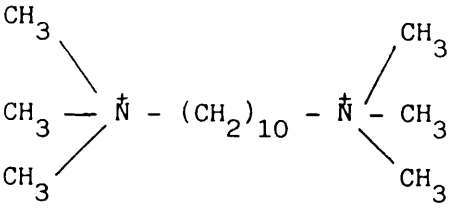
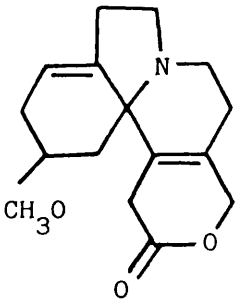
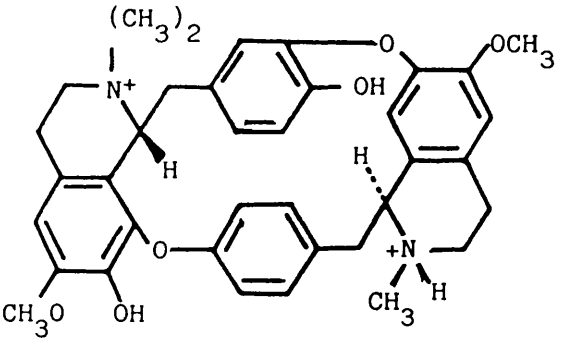


Decamethonium

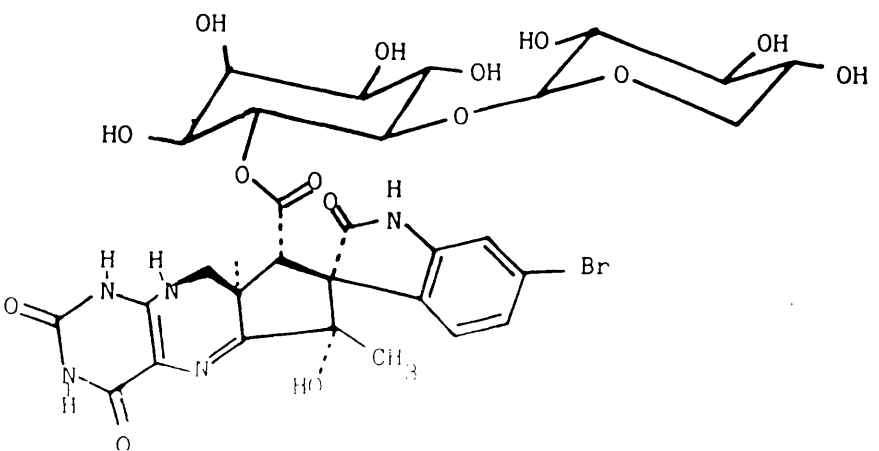


Dihydro-ß-erythroidine



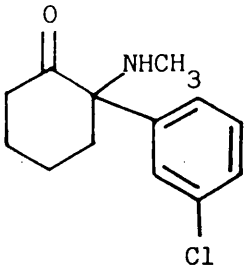
d-Tubocurarine

Neosurugatoxin

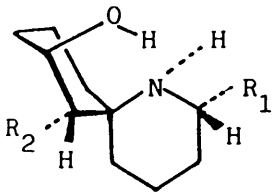


Drugs specific for ion channels

Nicotinic Acetylcholine receptor



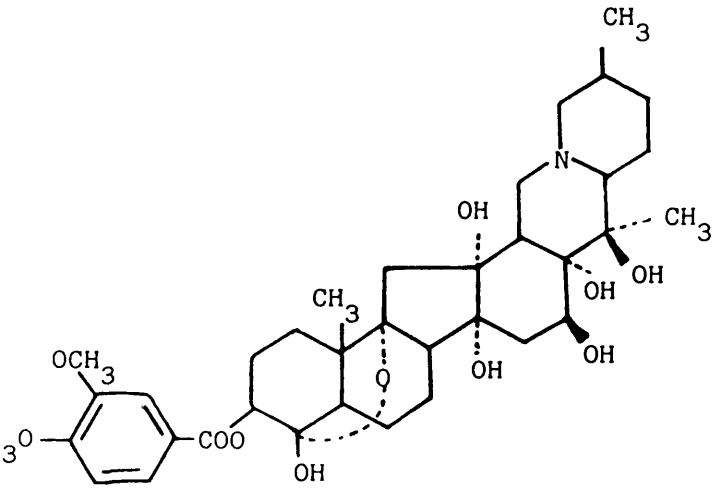
Ketamine hydrochloride



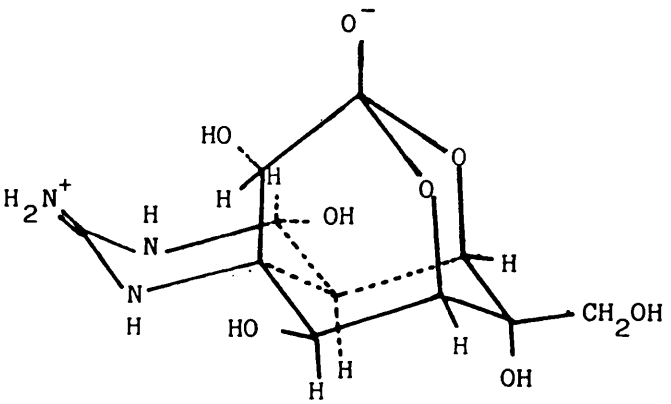
Histrionicotoxin (HTX)

	R_1	R_2
HTX	$CH_2-CH=CH-C\equiv CH$	$CH=CH-C\equiv CH$
H_{12}HTX	$CH_2-CH_2-CH_2-CH_2-CH_3$	$CH_2-CH_2-CH_2-CH_3$

Voltage sensitive sodium channel

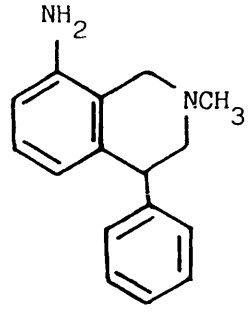


Veratridine

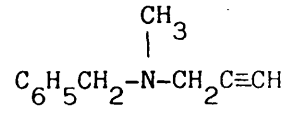


Tetrodotoxin

Others



Nomifensine (DA uptake inhibitor)



Pargyline
(monoamine oxidase inhibitor)

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RAPID IMPORTANT PAPER

NEOSURUGATOXIN BLOCKS NICOTINIC ACETYLCHOLINE RECEPTORS IN THE BRAIN

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ABSTRACT

Neosurugatoxin, a neurotoxin isolated from the Japanese ivory mollusc (Babylonia japonica) is a nicotinic antagonist with a specificity towards ganglionic nicotinic receptors. At low concentration (5×10^{-8}M) neosurugatoxin inhibited the release of [^{3}H]dopamine evoked by 1,1-dimethyl-4-phenylpiperazinium (DMPP) from rat striatal nerve terminals, without affecting the response to K^+-depolarisation. In contrast, αbungarotoxin did not antagonise the action of DMPP. Neosurugatoxin also inhibited [^{3}H] nicotine binding to rat brain membranes but had no effect on [^{125}I]αbungarotoxin binding to the same tissue preparation. These results support the view that functional nicotinic receptors in the CNS resemble ganglionic nicotinic receptors. Neosurugatoxin has considerable potential as a useful probe for such receptors in the brain.

There is an increasing acceptance of the presence of nicotinic acetylcholine receptors in vertebrate CNS and autonomic ganglia. However, progress in understanding the particular functions of these receptors and how they relate to the well-characterised peripheral nicotinic receptor has to some extent been hampered by lack of availability of suitable probes. The snake toxin, αbungarotoxin, that has contributed so much to our understanding of nicotinic receptors in electroplaques and at the neuromuscular junction (Conti-Tronconi & Raftery, 1982) has been applied to the study of the neuronal receptor. There is considerable evidence, including immunological crossreactivity between brain αbungarotoxin binding proteins and peripheral nicotinic receptors (Norman et al., 1982; Wonnacott et al., 1982) that this toxin may bind selectively to a population of neuronal nicotinic receptors. However, its usefulness has been questioned, particularly in view of its ineffectiveness as an antagonist of many neuronal nicotinic responses (see Barnard & Dolly, 1982).

We have sought to complement our binding studies by developing a functional assay system for nicotinic receptors in which we measure the presynaptic nicotinic facilitation of [^{3}H]dopamine release from striatal nerve terminals (Mills & Wonnacott, 1984; Rapier et al., 1985). In such a system we have demonstrated that an antiserum raised against the nicotinic receptor

purified from rat muscle inhibits the nicotine-evoked release of [^{3}H]dopamine (Mills & Wonnacott, 1984) suggesting some degree of identity between peripheral receptors and this group of central nicotinic receptors. However, in apparent contrast αbungarotoxin did not have a pronounced effect on the striatal preparation whereas the ganglionic antagonist mecamylamine completely blocked the nicotine-evoked response (Mills & Wonnacott, 1984). Recent reports of a neurotoxin isolated from the Japanese ivory mollusc (Babylonia japonica) and called neosurugatoxin (Kosuge et al., 1982; Hayashi et al., 1984) which may be a specific antagonist of some neuronal nicotinic receptors, prompted us to compare its action in our functional system with its effect in conventional binding assays.

MATERIALS & METHODS

D,L-[N-methyl ^{3}H]nicotine (73.7 Ci/mmol) was purchased from NEN Radiochemicals Ltd. (Southampton, Hants., U.K.) [^{125}I]Na and 7,8[^{3}H]dopamine (46 Ci/mmol) were purchased from Amersham International (Amersham, Bucks., U.K.) αbungarotoxin was from the Boehringer Corp. (Lewes, Sussex, U.K.) and was iodinated as previously described (Wonnacott et al., 1980).

Pure crystalline neosurugatoxin was a gift from Professor T. Kosuge. The toxin (250μg) was dissolved in 0.12 ml of dimethylsulphoxide and diluted to 3.0 ml with water. L-nicotine was from the Sigma Chemical Co. (Poole, Dorset, U.K.), 1,1-dimethyl-4-phenylpiperazinium (DMPP) was from Aldrich Chemical Co. Ltd., (Gillingham, Dorset, U.K.) and all other reagents were from BDH Chemicals Ltd. (Poole, Dorset, U.K.) Drugs and toxins were serially diluted in perfusion medium or assay buffer just prior to use.

Preparation and perfusion of synaptosomes

Synaptosomes from rat striata were prepared as described by Mills & Wonnacott (1984), the final suspension having a protein concentration of 3mg/ml. The synaptosomes were incubated with [^{3}H]dopamine (0.1μM; 15 Ci/mmol) for 4 min at 37°C (Rapier et al., 1985). Samples (250μl) of the synaptosome suspension were transferred to Whatman GFF filters positioned in the perfusion chambers (modified Millipore glass filter units) and perfused with modified Krebs medium at a constant rate (9ml/h).

After a 40 min washout period, depolarising pulses (100μl) of KCl and the nicotinic agonist DMPP were applied in the perfusion medium, allowing a 20 min interval between successive pulses. Continuous collection of consecutive 0.2 ml fractions of the perfusate was achieved by using an LKB Redirac fraction collector; radioactivity was counted after addition of 3 ml of Aqualuma (LUMAC B.V. Ad Schaesberg, The Netherlands) in a Packard scintillation spectrometer (counting efficiency 40%).

In each experiment 3 perfusion chambers were operated in parallel: one served as a control without toxin and the second as the test system. In the third spontaneous release throughout the perfusion period was measured and this was subtracted from the transmitter release profiles of the control and test systems.

Radioligand binding assays

A crude membrane fraction (P_2) was prepared from rat brain cortices, and reconstituted in 50mM-phosphate buffer, pH 7.4, containing 0.1mM-PMSF and 0.01% (w/v) sodium azide, to give a protein concentration of approximately 10mg/ml. [^{3}H]nicotine binding to this preparation was performed essentially as described by Romano & Goldstein (1980). The tissue was diluted five-fold in 50 mM-HEPES buffer, pH 7.4, containing NaCl (118mM), KCl (4.8mM), $CaCl_2$ (2mM), $MgSO_4$ (2mM). Samples (0.25ml) were incubated with 40nM-[^{3}H]nicotine for 60 min at 22°C, in the presence and absence of 10^{-4}M-L-nicotine. Bound radioligand was separated by filtration at 4°C.

[^{125}I]αbungarotoxin binding to P_2 membranes was assayed by the method of Schmidt (1977). The fraction was diluted ten-fold in 50 mM-phosphate buffer, pH 7.4, and aliquots (0.5 ml) were incubated with 1 nM-[^{125}I]αbungarotoxin for 60 min at 22°C, in the presence and absence of 2.5×10^{-7}M-αbungarotoxin. Bound radioligand was separated by centrifugation (2 min at 10,000g; MSE Microcentaur bench centrifuge); the pellet was washed once with 1.5 ml ice-cold phosphate-buffered saline.

Drugs were incubated with the membrane fraction for 10 min prior to the addition of radioligand. Non-specific binding (excess cold ligand) was subtracted; under the conditions used specific binding was 44.7 ± 8.8 fmol/mg and 9.4 ± 1.1 fmol/mg protein for [^{3}H]nicotine and [^{125}I]αbungarotoxin respectively (mean ± S.E.M. for 3 membrane preparations).

RESULTS

Neosurugatoxin greatly diminished the DMPP-evoked release of [^{3}H]dopamine from striatal synaptosomes (Fig. 1). In the absence of toxin (Fig. 1a), low doses of the ganglionic nicotinic agonist DMPP (5×10^{-6}M) or KCl (28mM) elicit sharp peaks of released radioactivity. After perfusing with neosurugatoxin (5×10^{-8}M) for 20 min the DMPP-evoked response was reduced: peaks 1 and 3 (Fig. 1b) represent only 21% and 15% respectively of the corresponding peaks in the normal control (Fig. 1a). The average inhibition of DMPP-evoked release by 5×10^{-8}M-neosurugatoxin was 59 ± 8% (mean ± S.E.M. of duplicate determinations from 3 independent experiments). K^+-evoked release was not significantly affected. After washing with normal perfusion medium without neosurugatoxin the response to DMPP was gradually restored (83% after 20 min washing, 109%

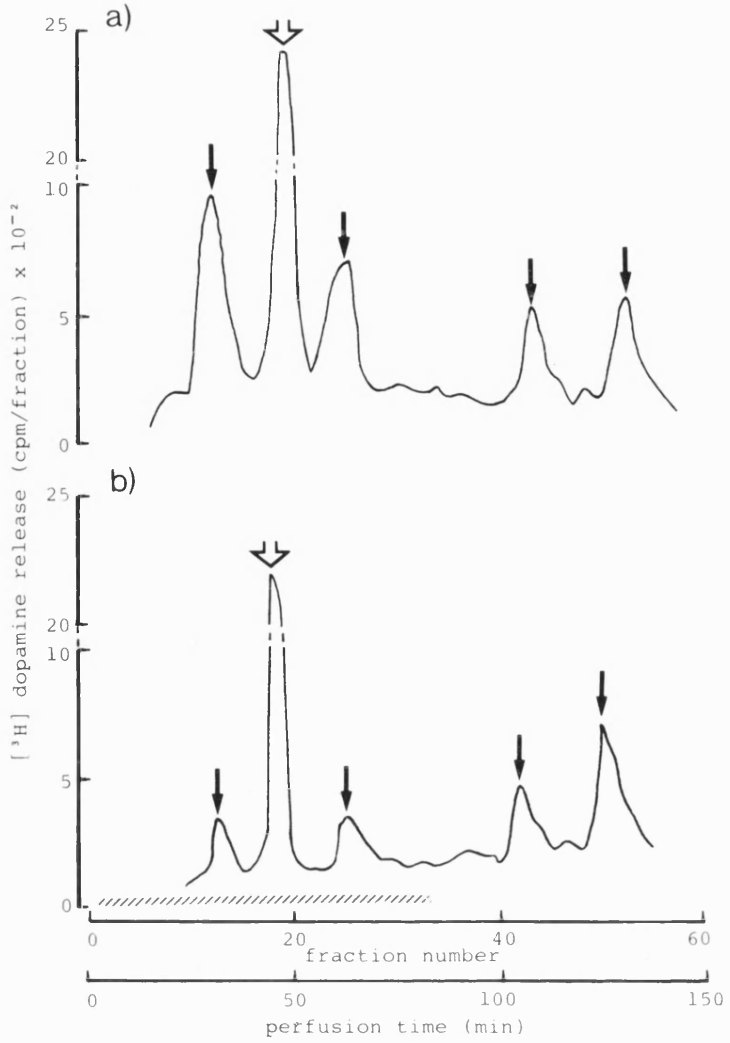

Fig. 1 Release of [³H]dopamine from striatal synaptosomes.

Synaptosomes were preincubated with [³H]dopamine and perfused with a) normal medium, or b) medium containing 5 x 10^{-8}M neosurugatoxin for 90 min (////). Pulses (100µl) of DMPP (5 x 10^{-6}M; ↓) or K^+ (28 mM, ⇓) resulted in responses indicated by the arrows.

after 40 min; Fig. 1b). Exposure to αbungarotoxin (5 x 10^{-8}M) under identical conditions to those of the neosurugatoxin experiments, was ineffective in inhibiting DMPP-evoked [³H]dopamine release. Neosurugatoxin also inhibited the

binding of [^{3}H]nicotine to rat brain membranes (Fig. 2a). L-nicotine displaced radio-labelled nicotine but αbungarotoxin was largely ineffective up to 10^{-4}M. In contrast, when these ligands were tested against [^{125}I]αbungarotoxin binding to the same tissue (Fig. 2b) αbungarotoxin was the most potent competitor and neosurugatoxin was without any effect, at concentrations up to 10^{-5}M. However, nicotine and DMPP were also good inhibitors of [^{125}I]αbungarotoxin binding.

DISCUSSION

The presynaptic nicotinic facilitation of dopamine release from striatal nerve terminals (Chesselet, 1984) provides a functional assay for a population of nicotinic receptors in the brain. This system has been partially characterised pharmacologically (Giorguieff et al., 1977). We have previously demonstrated that antibodies raised against the nicotinic receptor from skeletal muscle block the cholinergic facilitation of dopamine release from striatal synaptosomes (Futerman et al., 1982; Mills & Wonnacott, 1984) but αbungarotoxin was not very effective in our preparations. We have now greatly improved the sensitivity of our synaptosomal perfusion system (Rapier et al., 1985) and

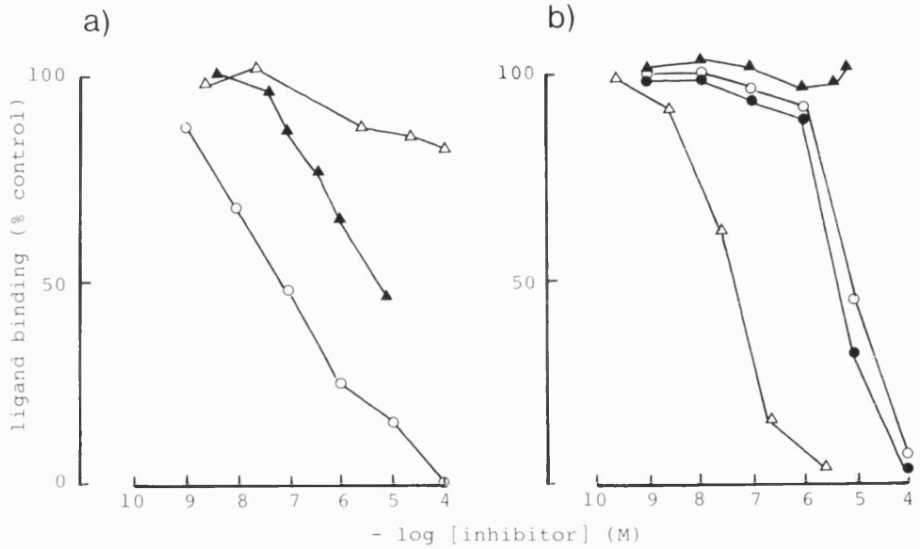

Fig. 2 Effect of drugs and toxins on a) [^{3}H]nicotine and b) [^{125}I]-αbungarotoxin binding to rat brain membranes.

Membranes (P_2 fraction) were preincubated with L-nicotine (○—○), DMPP (●—●), αbungarotoxin (△—△) or neosurugatoxin (▲—▲) at the final concentrations indicated for 10 min before addition of radioligand and assay as described in the Materials & Methods section. Data from a representative experiment are presented as a percentage of specific binding; each point is the mean of triplicate determinations, with variance of less than 10%.

clear repetitive responses are obtained to drugs in the μmolar range and below (Fig.1a). In this system the response to the nicotinic agonist DMPP is greatly diminished following exposure of the synaptosomes to neosurugatoxin whereas such treatment is without effect on the Ca^{++}-dependent, K^{+}-stimulated release (Fig. 1b). These findings strongly suggest that the toxin specifically blocks the presynaptic nicotinic facilitatory receptor. αBungarotoxin was without effect under identical conditions. Continuous perfusion with αbungarotoxin would be expected to achieve a more effective blockade than the brief exposure previously employed (Mills & Wonnacott, 1984). This clearcut negative result would seem to confirm that αbungarotoxin is not an antagonist at this nicotinic receptor. This conclusion is in agreement with the study of Misgeld et al. (1980) in which 6 x 10^{-7}M-αbungarotoxin was without effect on the intrinsic cholinergic excitation in striatal slices, although de Belleroche & Bradford (1978) reported a modest inhibition (38%) of acetylcholine - evoked [^{3}H]dopamine release from striatal slices by 1.8 x 10^{-7}M-αbungarotoxin (see Mills & Wonnacott, 1984).

It has been reported that surugatoxin and neosurugatoxin (Kosuge et al., 1982), are potent antagonists of nicotinic receptors in sympathetic and parasympathetic ganglia but not at the neuromuscular junction (Hayashi & Yamada, 1975; Brown et al., 1976; Ascher et al., 1979). Indeed in the study of Hayashi and Yamada (1975) surugatoxin specifically inhibited the actions of DMPP on blood pressure and ganglionic transmission in the cat and DMPP-evoked contraction of the guinea pig ileum. The latter action has been reproduced with neosurugatoxin itself (Hayashi et al., 1984). In this case, the toxin may act at presynaptic nicotinic receptors that facilitate transmitter release from myenteric varicosities (Briggs & Cooper, 1982; White, 1982), since the post-ganglionic response to acetylcholine (via muscarinic receptors on the muscle surface) was unaffected by neosurugatoxin (Hayashi er al., 1984). The present study is the first report of the antagonism by neosurugatoxin of nicotinic receptor function in the brain, and supports the view that many central nicotinic receptors may be ganglionic in character (see Schmidt et al., 1980).

In agreement with Hayashi et al. (1984) we find that neosurugatoxin inhibits [^{3}H]nicotine binding to brain membranes (Fig. 2a). Quantitative assessment of the inhibition in our experiments was difficult because of the instability of neosurugatoxin in aqueous solution (Kosuge et al., 1982). What is clear however, is its differential effect on binding of [^{3}H]nicotine and [^{125}I]αbungarotoxin to brain membranes (Fig. 2); the binding assays were performed in parallel using the same serial dilutions of neosurugatoxin. Nicotinic antagonists generally are very poor inhibitors of [^{3}H]nicotine binding to nervous tissue (Romano & Goldstein, 1980; Marks & Collins, 1982; Costa & Murphy, 1983), and we find that αbungarotoxin has little effect on [^{3}H] nicotine binding, in agreement with other studies. Neosurugatoxin, however,

shows strong inhibition of binding and is the first antagonist to be shown to have potent effect at the nicotine binding site. In contrast, although nicotine and DMPP are effective competitors for [^{125}I]αbungarotoxin binding sites (Fig. 2b; Marks & Collins, 1982), neosurugatoxin is without effect. Such data do not preclude binding of [^{3}H]nicotine and [^{125}I]αbungarotoxin at distinct sites on the same oligomers, which would be consistent with the inhibition of nicotine-evoked release by an antiserum that crossreacts with the αbungarotoxin binding component from rat brain (Mills & Wonnacott, 1984). Irrespective of the precise relationship of the binding sites, our results are consistent with the widely held view that αbungarotoxin is a poor antagonist of known nicotinic cholinergic function in the CNS. Conversely, neosurugatoxin shows considerable promise as a potent, selective probe for functional central nicotinic receptors.

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